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Acta Pathologica et Microbiologica Scandinavica is intended for the prompt publication of original research in the fields of pathology, microbiology, and immunology. It is included in Current Contents, Excerpta Medica, and Medlars.

Acta Pathologica et Microbiologica Scandinavica is a nonprofit-making scientific journal. Since 1924, it has been published by the Scandinavian Societies for Medical Microbiology and Pathology. It appears in three sections: Section A: Pathology, Section B: Microbiology, and Section C: Immunology.

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SUPPLEMENTS

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ENZYME LINKED IMMUNOSORBENT ASSAY (ELISA) FOR DETERMINATION OF IgG ANTIBODIES TO HUMAN CYTOMEGALOVIRUS

ISRAEL SAROV[1] PAUL ANDERSEN[2] and HANS KERZEL ANDERSEN[2]

Virology Unit Faculty of Health Sciences and Soroka Medical Centre Ben Gurion University of The Negev Beer Sheva Israel[1] and Institute of Medical Microbiology University of Aarhus Aarhus Denmark[2]

Sarov I Andersen P & Kerzel Andersen H Enzyme linked immunosorbent assay (ELISA) for determination of IgG antibodies to human cytomegalovirus Acta path microbiol scand Sect B 88 1– 9 1980

A solid phase enzyme linked immunosorbent assay (ELISA) for determination of IgG antibodies to cytomegalovirus (CMV) is described The assay uses purified CMV and extracts of CMV infected cells as antigen Antigens were desiccated onto the bottom surface of polystyrene microcuvettes The antibodies bound to the antigens were assayed by anti IgG alkaline phosphate conjugate followed by addition of the enzyme substrate Titration curves have been obtained from the sera of 35 blood donors and of [illegible] A with those obtained by complement [illegible] oth purified CMV and extracts of CMV [illegible] was of value particularly in those sera which show high reactivity against control antigen The ELISA technique described is approximately 412 to 548 times more sensitive than the CF test when purified CMV or extracts of CMV infected cells respectively are used as antigens No significant heterotypic rise to CMV was observed by ELISA in three sets of sera with seroconversion to herpes simplex virus The ELISA technique gives objective results is easily performed and may be adaptable as a routine test both for serological diagnosis of CMV infection and for screening of the general population

Key words CMV ELISA CF

I Sarov Virology Unit Faculty of Health Sciences and Soroka Medical Centre Ben Gurion University of The Negev Beer Sheva Israel

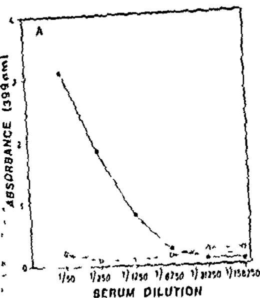
Received 6 vi 79 Accepted 20 viii 79

Human cytomegalovirus (CMV) is a major viral cause of birth defects which include mental retardation blindness and deafness (26) In the normal human population CMV infections are generally subclinical and the virus remains in a latent potentially infectious form CMV infect[illegible] [illegible] with CMV may constitute a serious problem for immunosuppressed individuals such as recipients of organ transplants and cancer patients (20)

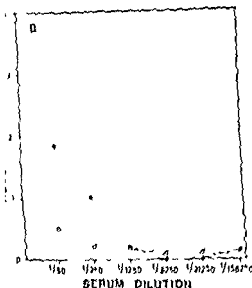
The accurate diagnosis of CMV infection is of major concern to medicine Currently, the methods used most frequently are based on virus isolation and detection of antibodies to CMV by the complement fixation (CF) test the indirect hae magglutination assay (IHA) (13) or the indirect fluorescent antibodies assay (IFA) (8) Recently, an indirect solid phase radioimmunoassay (RIA) has been developed for measurement of antibodies to CMV (17) The sensitivity of the RIA accounts for its widespread growth as an assay technique However this technique possesses certain disadvan tages viz. the short self life of reagents and the

SUPPLEMENTS

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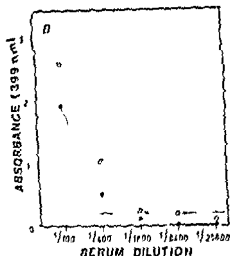
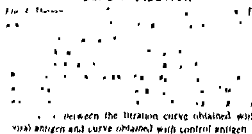


Fig. 2 Titration curve of a positive serum on purified CMV antigen (A) and extracts of CMV infected cell antigen (B) showing high control antigen reactivity giving a false negative result

Solid lines: sera tested on CMV antigens

Dotted lines: sera tested on control antigens



... between the titration curve obtained with viral antigen and curve obtained with control antigen

...13 000 by ELISA with purified CMV antigen (Fig. ...)

As already mentioned, we have chosen arbitrarily ... end point titre for the purified CMV antigen as the intersection of the titration curve of the viral antigen with an arithmetic average of the background. Since we are detecting antibodies by an enzymatic reaction, we wished to veryfy our end-point determination by prolonging the reaction time. Table 1 demonstrates this experiment performed with purified CMV as antigen. When absorbance readings (A_{399}) were taken 2.5 hours after substrate addition, one obtained approximately the same titre as after 43 hours, viz. 1.9 × 10[4]. With time, the absorbance of each serum dilution approached the maximum reading of the LKB apparatus, 9.9. Beyond the end point (in this case

potential health hazard of the radiation A promis ing alternative to RIA appears to be the enzyme linked immunosorbent assay (ELISA) developed recently by a number of investigators (2, 9–11 27) Recent studies have shown that this method is suitable for antibody assay in diagnostic microbio logy (3, 5 6 15 16 18 23)

In preliminary studies *Voller & Bidwell* (27) and *Castellano et al* (7) have described a micro ELISA method for CMV IgG antibodies using commercial CMV antigens and extracts of CMV infected cells *Schmitz et al* (25) developed ELISA for determina tion of CMV IgM antibodies by applying nuclei of CMV infected cells as antigen The present study reports the development of ELISA for detecting CMV IgG antibodies using both purified CMV and extracts of CMV infected human embryonic fibro blasts (HEL) as antigens We have compared the sensitivity of this CMV IgG ELISA with that of the complement fixation test (CF)

MATERIALS AND METHODS

Sera Eighty one sera were examined including 10 from 5 patients with CMV infection 6 from 3 patients with herpes simplex virus (HSV) stomatitis and 30 from patients with other viral diseases as well as 35 healthy blood donors All sera were inactivated at 56 °C for 30 min and stored at −20 °C until use

Viral antigens Highly purified CMV strain AD 169 was obtained from the supernatant fluids of infected HEL cells by successive zone and density centrifugation as described by *Sarov & Abady* (24) The infectivity of the viral preparations was determined by the method of *Plummer & Benyesh Melnick* (22) A virus suspension of 1 OD $_{260nm}$ gave 10^7–10^8 PFU/ml The viral bands were dialysed overnight against phosphate buffered saline (PBS) pH 7 2 and adjusted to 20 µg of protein per ml as determined by *Lowry et al* (19) The purified viral antigens were divided into small aliquots and stored at −70 °C Control antigen to the purified virus was obtained by processing supernatant fluid from fibroplasts of the same line in the same way as that used to obtain purified CMV

CMV HEL antigen was produced by infecting cells in Roux bottles with 1 PFU/cell of CMV strain AD 169 After 9–12 days the infected cells were washed with 10 ml of PBS frozen and thawed 3 times in 3 ml of coating buffer pH 9 6 (27) Cell debris was removed by centrifugation at 300 *g* for 10 min The supernatant was divided into small aliquots and stored at −70 °C Control antigen was prepared in the same way from non infected cells and stored at −70 °C

ELISA The procedure is a modification of that described previously by *Voller & Bid ell* (27) Twenty five microlitre drops of the various antigens were air dried overnight at room temperature in polystyrene cuvettes (LKB) The dried antigens were washed 3 times with phosphate buffered saline pH 7 4 conta 0 05% Tween 20 (PBS T) 0 1 ml of four (or five) serum dilutions in PBS T was added and the sampl duplicate were incubated for 3 hours at room temp ture under continuous shaking in a Marius shaker cuvettes were washed 3 times with PBS T and 0 1 n conjugated swine antibodies against human IgG (OR Diagnostica – Finland) was added and incub overnight with shaking at room temperature conjugate was used at dilution 1 400 in PBS T The day the cuvettes were washed 3 times with PBS T 0 1 ml of p nitrophenyl phosphate substrate (Sigma) concentration of 1 mg/ml in 10% diethanolan buffer pH 9 8 was added The cuvettes were incub at room temperature for 1 hour and the reaction stopped by adding o 5 ml of 0 3 N NaOH Absorbanc 399nm was measured in an LKB 7400 calcula absorptiometer Within a linear absorbance range apparatus was adjusted to express the absorbance values between 0 and 9 9 Cuvettes containing disti water were used as reference blanks Known positive negative sera were included in each experiment as as 6 cuvettes containing only serum diluent instead of serum An average background adsorption level determined and this value was substracted from e sample before plotting the curves

Other serological methods The complement fixat test (CF) was performed as described previously (1)

RESULTS

Determination of Serum Titre

Fig 1 demonstrates the titration curves of kno CMV positive sera tested on purified virus (F 1A) extracts of CMV HEL (Fig 1B) and cont antigens (dotted lines) For the purified virus have chosen the intersection of the titration cu using viral antigen with the background line as t titre of the serum The background line marks t absorbance value at 399nm(A_{399}) obtained with t serum diluent on viral antigen instead of ser (average of 6 determinations) When the standa CMV positive serum was titrated in five expe ments using two different batches of purified vir the maximum difference in titre was in the two-fc range

When extracts of CMV HEL (Fig 1B) were us as antigen we took the intersection between t titration curve obtained with viral antigen and t corresponding curve obtained with control HI antigen as the viral titre

While most of the sera react only slightly wi control HEL antigen (at most the two first dilutio of test sera) we found a false negative result in transplant patient in whose serum high non speci reactivity with control HEL antigen was detect (Fig 2 A) This serum has a *titre of 16 by CF* ar

TABLE 2 *CMV Antibody Titre of Paired Sera of Patients with Seroconversion to Herpes Simplex Viruses*

Donors	Age (years)	Date	HSV titre[a]	CMV ELISA	
				purified CMV	CMV HEL ag
MT	4	17-5-76	4	<100	<100
		9-6-76	32	<100	<100
TT	6	21-5-76	4	<100	<100
		9-6-76	64	<100	<100
SM	5	10-5-76	4	<100	<100
		31-5-76	32	<100	<100

a) Determined by complement fixation test (1)

protein per ml of CMV HEL antigen resulted in no significant change in the sensitivity With lower antigen concentration (10–50 µg protein per ml) the assay sensitivity was decreased and particularly the discrimination between positive and negative sera (data not shown) A concentration of 200 µg protein per ml was chosen for use in further experiments The highest sensitivity with purified CMV antigen was obtained with the highest concentration of CMV antigen tested (20 µg protein per ml) At lower antigen concentration (5 and 1 25 µg/ml) the assay sensitivity was markedly decreased

Specificity of the Assay

To exclude the possibility of heterologous cross reactivity between antigens of CMV and herpes simplex virus we used both purified and CMV HEL antigens to test the titres of three paired sera which showed seroconversion to herpes simplex type *1* Table 2 shows that when there is a specific rise in complement fixing (CF) antibodies against herpes simplex virus no corresponding rise was found by ELISA against either purified CMV or extracts of CMV HEL antigens

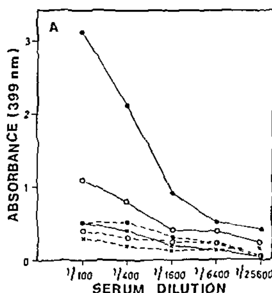
TABLE 3 *Comparison of CF and ELISA Titres of Patients with Acute CMV Infections*

Sera No	Clinical diagnosis	Age (years)	Time from first serum (days)	CF	Antibody titres ELISA		Absorbance (399 nm) Dil 1 250	
					purified CMV	CMV HEL Ag[a]	purified CMV	CMV HEL ag[a]
13473	Reticulo		0	16	12 500	850	2 0	0 7
13951	sarcoma	57	81	256	80 685	12 500	3 0	1 9
13746	Renal		0	64	9 300	1 800	0 9	1 0
2262	transplant	46	85	256	32 000	6 250	2 6	2 0
2836	Renal		0	8	3 375	1 700	0 6	0 7
3009	transplant	33	30	128	16 250	25 000	2 8	2 0
2837	Renal		0	8	5 625	4 250	1 2	1 2
3027	transplant	41	36	256	25 000	30 000	2 2	2 6
3001	Renal		0	4	50	50	0 1	0 0
3002	transplant	33	13	32	250	250	0 2	0 2

a) CMV HEL [illegible]

TABLE 1 *Influence of Reaction Time on CMV ELISA Titre Determination on Purified CMV Ag*

Reaction time (hours)	Serum dilutions	Absorbance values (399 nm)							
		50	250	1,250	6,250	31,250	156,250	Background	CMV ti
2½		3 10	1 90	0 90	0 25	0 10	0 10	0 17	1 6×10
9		7 20	4 30	1 80	0 50	0 12	0 06	0 21	1 8×10
19		9 90	7 58	3 48	0 71	0 10	0 03	0 28	1 9×10
30		9 90	9 30	4 60	1 06	0 08	0 06	0 37	1 9×10
43		9 90	9 90	5 60	1 32	0 10	0 05	0 44	1 9×10



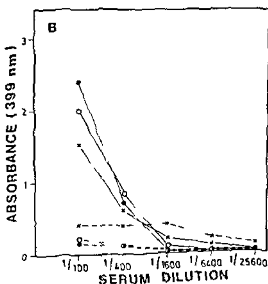


Fig 3 Serum titration curves showing effect of var
concentration of purified CMV (A) and extracts of CM
HEL antigen (B) dried onto microcuvettes
A) Purified CMV antigen ● 20 μg/ml ○ 5 μg/ml
1 25 μg/ml antigen concentration
B) Extracts of CMV HEL antigen (B) ● 800 μg/
○ 200 μg/ml *X 50 μg/ml*
Solid lines, sera tested on CMV antigens
Dotted lines sera tested on control antigens

dilution 1/31250) there was no net increase i A_{399} value with time, which shows that t *endpoint is indeed reached* We have performe measurements similar to those described above o 36 sera which include CMV patients and bloo donors, *and similar results were obtained* How ever, it should be noted that prolongation of th *reaction time is not suitable for discriminatio* between low positive and negative sera, since som *of the negative sera with time showed a non-specif positive reaction Confirmation of the viral titre b* prolonging the reaction could be performed wit CMV HEL antigen also but in that case th *reaction time should be prolonged for no more tha* 24 hours on account of the high rise in backgroun activity

Determination of Optimal Antigen Concentration

The optimal concentration of various antigen was determined by titration of one positive and on negative serum against known CMV HEL antige (Fig 3 B) and purified CMV antigen (Fig 3 A Using an antigen concentration of 200 to 800 μ

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a) Determined by complement fixation test (1)

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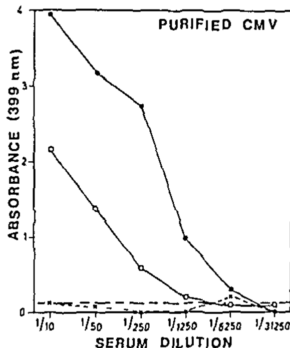
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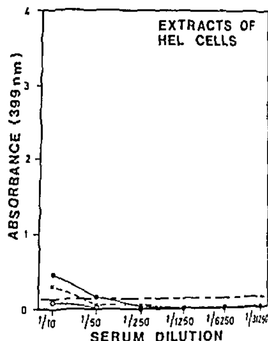
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13851	sarcoma	57	81	256	80 685	12 500	3 0	1 9
13746	Renal		0	64	9 300	1 800	0 9	1 0
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2836	Renal		0	8	3 375	1 700	0 6	0 7
3009	transplant	33	30	128	16 250	25 000	2 8	2 0
2837	Renal		0	8	5 625	4 250	1 2	1 2
3023	transplant	41	36	256	25 000	30 000	2 2	2 6
3001	Renal		0	4	50	50	0 1	0 0
3002	transplant	33	13	32	250	250	0 2	0 2

a) CMV HEL ag extracts of CMV HEL cells antigen.





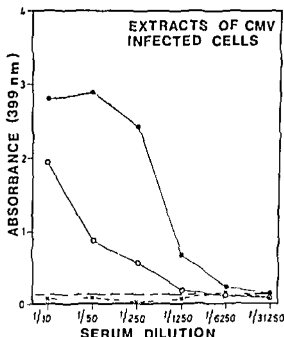


Fig 4 Diagnosis of CMV infection paired sera of a 3 year old renal transplant patient showing a rise in ELISA titre with purified CMV and extracts of CMV and HEL as antigens
Solid lines open symbols first serum
Solid lines solid symbols second serum taken 36 days later
Dotted lines negative sera

Patients with CMV Infections

The ELISA results obtained for paired sera of 5 CMV patients (Table 3) were compared with those obtained by CF Fig 4 illustrates ELISA results obtained for one serum pair from a CMV patient with both purified and CMV HEL antigens With both antigens a diagnostic titre rise could be demonstrated (purified CMV antigen 5625 to 25 000 CMV HEL antigen 4250 to 30 000)

In both procedures four fold or greater rises in titre were observed for each of the serum pairs (Table 3) The ELISA gave titres much higher than those obtained by CF For example for one CMV patient, a titre of 80 685 was obtained by ELISA as compared to 256 by CF It should be noted that for four of five of our CMV patients a difference in absorption at 399nm between acute and convalescent sera was found at a serum dilution of 1 250 when the reaction was carried out for 1 hour (standard conditions) Thirty sera of 15 patients with unrelated diseases were examined and none showed significance CMV titre differences between acute and convalescent sera by ELISA or by CF

Blood Donors

A sample of 35 sera of presumably healthy blood donors was evaluated for CMV antibody titre by ELISA and by CF using both purified and CMV HEL antigens

a) Negative sera Eighteen out of these 35 sera were negative by the CF test (titre < 4) All 18 were negative by ELISA (titre < 100) using CMV HEL antigens These sera had control antigen curves either identical with or slightly higher than viral antigen curves Fourteen of the 18 were negative by ELISA using purified CMV antigen However, 4 of the 18 showed a higher reactivity against purified antigen than against control HEL

TABLE 4 *Comparison of CF and ELISA Titres of CMV Positive Blood Donors*

Sera No	Antibody titres		
	CF	ELISA	
		Purified CMV	CMV HEL Ag
5538	4	2100	6400
5513	8	2600	2000
5512	8	3400	10 000
5519	8	3600	6400
5525	8	5300	5000
5926	8	5500	10 000
5925	8	6400	10 000
5511	16	3800	10 000
5666	16	5100	20 000
5712	16	8000	10 000
5772	16	9700	6400
5517	16	10 000	20 000
5536	16	15 000	4000
5515	32	5000	10 000
5514	32	18 000	6400
5734	64	13 000	20 000
5941	128	13 000	10 000
GMT[a]	15 4	6336	8419

[a] Geometric mean titre (GMT)

antigen giving titres of about 1/400 To investigate the poss bility that the low titre of about 1/400 obtained above in 4 of these sera represents a non specific reaction of the sera to the purified virus these sera were re-examined by including a protein supplement consisting of 1% bovine albumin in PBS The supplement was added to the antigen treated cuvettes for / hour at room temperature and subsequently washed off Two of these sera became completely negative whereas in the other two no change in titre was obtained

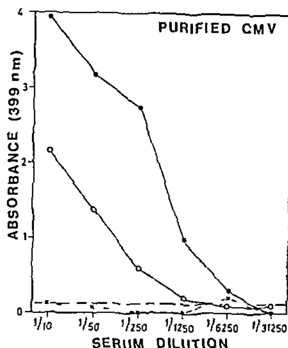
b) *Positive sera* Of the sample of 35 sera 17 were found positive by CF test All 17 were positive both when using purified CMV antigen and when using CMV HEL antigen Sera which showed low titres of CF also showed low titres by ELISA with purified CMV antigen (Table 4) and vice versa However the correlation between CF and Elisa was not complete and is less clear when CMV HEL antigen is used in ELISA (Table 4) The average geometric mean titre (GMT) for the CMV positive blood donors by CF is 15.4 whereas by ELISA a GMT 6336 and 8419 was obtained using purified CMV or CMV HEL antigen respectively

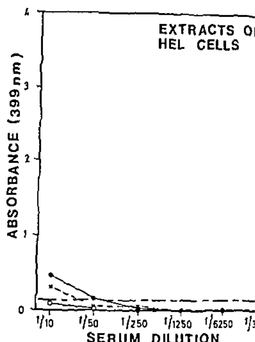
DISCUSSION

The present study demonstrates the applicability of the ELISA system as an additional serological tool for determination of CMV IgG antibodies Under our experimental conditions both purified and CMV HEL antigens were suitable as antigens (Table 3 Table 4) However a false negative reaction or error in titre determination may occur in those sera which possess very high reactivity against control HEL antigen (Fig 2) Purified CMV antigen could be of value particularly in those sera which contain high non specific reactivity against control HEL antigen (Fig 2 B)

There is no generally accepted formula for determination of serum titre by ELISA We have chosen the end point titre as the intersection of the titration curve with a background line (the A_{399} value of the serum diluent instead of serum) in the case of purified CMV antigen and the intersection of the titration curve on viral antigen with the corresponding curve on control antigen when crude antigen extracts of CMV infected cells are used In the RIA systems some investigators defined as a cut-off point › background ‹ + 2 (21) or + 3 (4) standard deviations Other establish a reference line of two times a background defined as the average of 12 replicates of viral antigens and 12 replicates of control antigen (17) and some determine titre as the last point at which there is no longer a ratio of at least 2 1 between counts bound to viral antigen and counts bound to control antigen (12) Since we are detecting antibodies by an enzymatic reaction we could confirm our assumption by prolonging the reaction time (Table 1) With time there is a gradual rise in the absorbance of each serum dilution showing antibody activity but no rise beyond the end point This experiment indicates that we have a proper method for calculating the ELISA titres

The ELISA system appears to be specific and no cross reactivity with herpes simplex virus was observed (Table 2) However many more samples of paired sera will have to be examined in order to determine whether the method is completely speci fic To examine further the specificity of ELISA Serum titrations were obtained for paired sera of 5 CMV patients using purified and CMV HEL antigen (Table 3) The results of ELISA titration showed complete agreement with those obtained by CF By both methods a significant rise in titre was obtained for each serum pair The specificity of ELISA test was analysed further on 35 presumably healthy blood donors using both purified and CMV HEL antigens (Table 4) These results were then compared with CF titres It was found that all the





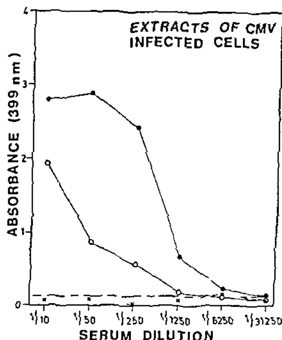


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Dotted lines negative sera

Patients with CMV Infections

The ELISA results obtained for paired sera of 5 CMV patients (Table 3) were compared with those obtained by CF Fig 4 illustrates ELISA results obtained for one serum pair from a CMV patient with both purified and CMV HEL antigens With both antigens a diagnostic titre rise could be demonstrated (purified CMV antigen 5625 to 25 000 CMV HEL antigen 4250 to 30 000)

In both procedures four fold or greater rises in titre were observed for each of the serum pairs (Table 3) The ELISA gave titres much higher than those obtained by CF For example for one CMV patient a titre of 80 685 was obtained by ELIS compared to 256 by CF It should be noted tha four of five of our CMV patients a differen absorption at 399nm between acute and con scent sera was found at a serum dilution of 1 when the reaction was carried out for 1 hour (s dard conditions) Thirty sera of 15 patients unrelated diseases were examined and none sho significance CMV titre differences between a and convalescent sera by ELISA or by CF

Blood Donors

A sample of 35 sera of presumably healthy bl donors was evaluated for CMV antibody titre ELISA and by CF using both purified and C HEL antigens

a) *Negative sera* Eighteen out of these 35 s were negative by the CF test (titre < 4) All were negative by ELISA (titre < 100) using C HEL antigens These sera had control anti curves either identical with or slightly higher th viral antigen curves Fourteen of the 18 w negative by ELISA using purified CMV antig However 4 of the 18 showed a higher reactiv against purified antigen than against control H

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17 sera which were positive by CF were also positive by ELISA

The sensitivity of the ELISA method is, however, up to 412 or 548 times the sensitivity obtained by the CF test This determinations is based on the assumption that a comparisin of geometric mean titre (GMT) can give an approximation of relative sensitivities of 2 techniques The GMT of the blood CMV positive donors is 15 4 by CF and 6336 and 8419 by ELISA when applying purified and CMV HEL antigens, respectively

A measure of the reliability of a test is its ability to discriminate between positive and negative sera With extracts of CMV HEL as antigen, all the 18 sera which were negative by CF (<4) were also found negative by ELISA (<100) Thus, with CMV HEL antigen, the ELISA system discriminates between positive and negative sera With purified CMV antigen 2 out of these 18 sera were found to be positive by ELISA (1/400) but negative by CF (<4) These latter two sera should thus be considered to be either false positive or as having a very low titre of CMV antibodies (1/400) which could be detected only by purified CMV antigen but not when using CMV HEL antigen It should be noted that for purified CMV it is difficult to prepare a real control antigen, and the control antigen prepared from supernatant of uninfected HEL does not contain any detectable protein (24)

Although it has been reported that CMV infected cells possess Fc receptors (12, 14) these were not found by ELISA, since the negative sera have either identical titration curves on viral and control antigen or in some cases higher control antigen values It is possible that our antigen preparations do not contain the Fc receptors, since the CMV HEL antigen consists of an extract of frozen and thawed cells from which cell debris has been removed by centrifugation

To summarize the present study demonstrates that ELISA CMV IgG can be considered as a potential sensitive test for use both in diagnostic laboratories and for population screening studies

This study was supported by the fellowship to one of us (I S) from the *European Molecular Biology Organization* (EMBO) and by the *Israel Academy of Sciences* Furthermore the study was supported by *P Carl Petersens Ford* Denmark This work was presented at the Fourth International Congress for Virology Aug 30 1978 The Hague The Netherlands

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AN EFFECTIVE, SELECTIVE MEDIUM FOR *YERSINIA ENTEROCOLITICA* CONTAINING SODIUM OXALATE

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Soltesz L V Schalen C & Mårdh P A An effective selective medium for *Yersinia enterocolitica* containing sodium oxalate Acta path microbiol scand Sect. B 88 11–16 1980

A medium (»Y« medium) is described which was more efficient for the isolation of *Yersinia enterocolitica* from experimentally infected faecal specimens than desoxycholate-citrate McConkey lactose sucrose urea (LSU) agar and Yersinia selective medium (Walter s medium) The »Y« medium consists of casein hydrolysate and peptone serving as carbon and energy sources A high selectivity is achieved by its contents of sodium oxalate and bile salts The oxalate suppresses growth of gram negative rods including members of the family *Enterobacteriaceae* and of *Pseudomonas* spp while the bile salts inhibit growth of gram positive bacteria In the few instances coliform rods *grew on the »Y«* medium they could easily be distinguished by their fermentation of lactose included in the medium and the fact that colonies of organisms were surrounded by an opaque zone of precipitated bile salts The most optimal condition for the isolation of *Y enterocolitica* from stools was achieved at incubation of the *»Y« medium at 29 °C for 2 days*

Key words *Yersinia enterocolitica* selective medium sodium oxalate stools diagnosis

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During recent years infections with *Yersinia enterocolitica* in Europe particularly with serotypes 3 and 9 (10 16 22) and in the US with serotype 8 (16 20 22) have been diagnosed by increasing frequency in man The organism has been associated with acute terminal ileitis, *lymphadenitis mesenterica* in children enteritis septicemia, erythema nodosum and arthritis It may also be isolated from pat[illegible]
(1 3 7 [illegible]
with [illegible]

In pu[illegible] generally grow as well on blood agar (HBA) desoxycholate-citrate (DC) lactose-sucrose urea (LSU) MacConkey (MC) agar (10 17) as on a [illegible] um being especially designed for isolation of this organism viz Yersinia selective medium (YS) (19) However on attempts to recover *Y enterocolitica* from faecal specimens differences in the isolation frequency when using these media are noted (10 11 19) Highly selective media seem to promote the recovery of *Y enterocolitica* (11 19). Incubation of stools at 4 °C for 3 weeks has also been demonstrated to increase the recovery frequency (4 5, 12 18 19)

Studies on nutritional requirements of *Y enterocolitica* resulting in the composition of a selective differentiating medium (»Y« medium) for this organism are presented The efficiency of the »Y« medium as compared with other media currently used for the isolation of *Y enterocolitica* for the recovery of the organism from experimentally infected faecal specimens is also described

Table 1 *Constituents of a New Selective Culture Medium for* Yersinia enterocolitica

Compound (designation/manufacturer)	Gram/litre distilled water
Bacteriological Peptone (L 37 Oxoid)	15
Casein Hydrolysate (L 41 Oxoid)	5
Lactose (Mallinkrodt)	10
Sodium Oxalate (BDH)	8
Sodium Desoxycholate (6504 Merck)	16
Sodium Chloride (6404 Merck)	5
Bile Salts (L 55 Oxoid)	5
Neutral Red (1369 Merck)	0 08
Bacto Agar (*B 140 Difco*)	20

Table 3 *Recovery of* Yersinia enterocolitica *from Experimentally Infected Faecal Specimens after 2 Days Incubation at 29 °C Using Five Different Media Designation of Media see Text*

Medium	Number of stools *infected* (N)	Reisolation of *Y enterocolitica* from no of stools n	% of N
MC	197	175	88 8
DC	204	184	90 2
YS	116	96	82 7
LSU	142	126	88 7
»Y«	224	223	99 5

RESULTS

Composition of the »Y« Medium

The composition of the »Y« medium is given in Table 1 The use of both peptone and casein hydrolysate as carbon sources was found to be essential *The casein hydrolysate seemed to promote* rapid growth while peptone permitted development of large colonies of *Y enterocolitica* (Table 2)

Selectivity and Recovery Frequency of Y enterocolitica *from Experimentally Infected Faecal Specimens on the »Y« Medium and Different Variants Thereof*

When inoculating faecal specimens on the »Y« medium growth of gram positive bacteria was almost completely inhibited by the bile salts The growth inhibitory effect on gram positive bacteria was clearly demonstrated in the tests using »Y« medium *to which bile salts had not been added* Sodium desoxycholate at the concentration used not only suppressed growth of gram positive bacteria but also that of most gram negative rods including *Proteus* and *Pseudomonas* spp Occasionally some colonies of gram negative rods other than *Y enterocolitica* appeared on the »Y« medium If present these bacteria could easily be differentiated from *Y enterocolitica* because they produced acid from lactose and/or precipitated the bile salts *which resulted in a red and/or opaque zone around* the colonies

In the comparative studies of experimentally infected stools in which dicarboxylic acids other than oxalate viz 1 ketoglutarate and succinate were used the highest recovery frequency of *Y enterocolitica* viz 100% as well as the largest average colony-diameter were found when oxalate was used In this respect HBA was almost as efficient When the medium was supplied with sodium 1 ketoglutarate or succinate only 80% and 90% respectively of the tested 40 strains were *recovered and the* average colony-diameter was smaller (Table 4)

In the comparative studies using increasing amounts of sodium oxalate a concentration of 1%

Table 2 *Number of 40 Strains of* Yersinia enterocolitica *Growing on the New Medium Containing Bacteriological Peptone and Casein Hydrolysate 15 and 5 g per Litre Respectively (Y) as Compared to the Same Medium with an Increased Concentration of* Bacteriolog cal Peptone 20 g per Litre (Y BP), or of Casein Hydrolysate 20 g per Litre (Y *CH) after 2 Days Incubation at 29 °C*[a] *Diameter of Colonies*

Medium	Mean number of colony forming units		Diameter of colonies (mm)	
	24 hours	48 hours	Mean	Range
Y	23	31	2 3	1 3–2 5
Y BP	17	30	2 3	1 2–2 6
Y-CH	22	28	1 7	1 3–1 9

[a] The inoculum used resulted in approximately 30 colony forming units as tested on blood agar plates

MATERIALS AND METHODS

Test Strains

Altogether 81 strains of *Y enterocolitica* all of human origin were used in this study Of the strains 42 were obtained from Prof *S Winblad* Malmo Sweden while 26 strains came from Dr *K Gaarslev* Statens Serum institut Copenhagen Denmark The remaining 13 strains studied were isolated from faecal specimens sent to the Bacteriological Laboratory University Hospital Lund Sweden These latter strains were identified as *Y enterocolitica* on the basis of colony morphology gram staining biochemical reactions (see below) which were in principle performed as recommended by *Nilehn* (11) and agglutination tests using antisera purchased from Statens Bakteriologiska Laboratorium Solna Sweden

Biochemical Reactivity of Test Strains

All 81 strains formed acid from L arabinose cellobiose D fructose D galactose D glucose D mannitol D mannose sorbitol sucrose and D trehalose at both 22 ° and 37 °C They all decarboxylated ornithin but not arginin and lysin They also hydrolysed urea and 2 nitrophenyl beta D galactoside (ONPG) and were positive in the metyl red test None of the 81 strains fermented adonitol dulcitol inulin lactose raffinose rhamnose or D xylose Phenylalanin was not deaminated Aesculin hydrolysis and H_2S production were never demonstrated The oxidase reaction was negative for all strains The Voges Proskauer test was positive at 22 ° but negative at 37 °C This was also true for fermentation of maltose The biochemical tests were read after 2 and 3 days incubation at the two temperatures indicated

Composition of the »Y« Medium and Different Tested Variants Thereof

The composition and the suppliers of the constituents of the recommended selective medium for *Y enterocolitica* are given in Table 1 The following batches of constituents were used Casein hydrolysate (acid) batch no 158 9783 and 23 711616 Bacteriological peptone batch no 1 816071 and 29 912579 D(+)-lactose batch no WCHS 86 711-1 sodium oxalate batch no 8 535837 neutral red batch no 6 010861 Bacto agar batch no 644091 and bile salts

In the comparative experiments described below 40 artificially infected specimens were used (see below) In some of these experiments the »Y« medium from which bile salts were omitted was used In other studies the sodium oxalate in the »Y« medium was replaced by either 1 ketoglutarate (Merck Darmstadt W Germany batch no 6323613) or sodium succinate (Merck batch no 8522485) which both were used in concentrations equimolar to that of oxalate viz 0 06 M The optimal concentration of sodium oxalate was assessed in experiments using the »Y« medium (Table 1) to which increasing concentrations [0 2 up to 2 0% (w/v)] were added In other experiments 0 06 M PBS instead of NaCl 85 g l^{-1} was used in the »Y« medium which otherwise was of the same composition as that described in Table 1 Finally the carbon sources in the »Y« medium viz Bacteriological peptone (15 g l^{-1}) and Casein hydrolysate (5 g l^{-1}) were in some tests replaced by either 20 g l^{-1} of Bacteriological peptone or by on[ly] Casein hydrolysate

Preparation of the »Y« Medium

The medium constituents were suspended in distil[led] water holding approximately 10 °C which was the[n] heated to boiling in order to achieve complete dissolution After sterilisation viz autoclaving at 121 °C for [..] minutes the medium was immediately cooled to 4[5-] 50 °C and the pH adjusted to 7 4 The medium was dispensed into plastic dishes in an amount of 22 25 [ml] per dish which were stored over blue gel in a plastic [bag] at room temperature until use

Other Media Tested

Horse blood agar (HBA) consisting of Blood agar base no 2 (Oxoid batch no 22 611 ED 2 127) supplemented with 4% defibrinated horse blood DC agar (Oxoid batch no 199984 DN 15 442) YS agar (see reference 19) (Merck batch no 6 393 193) and MacConkey agar (Difco batch no 646079) were prepared in accordance with the instructions of the suppliers and LSU agar according to those of *Juhlin & Ericson* (6)

Recovery Tests

In one series of experiments each of 224 faecal specimens were infected with one of altogether 40 strains of *Y enterocolitica* belonging to serotypes 3 (25 strains) 6 (2 strains) 8 (2 strains) and 9 (11 strains) The infected stools were cultured on the DC MC LSU YS and »Y« media The bacteria were suspended in isotonic phosphate buffered saline (PBS) pH 7 4 to a density of approximatively 10^{4-5} cells ml^{-1} as determined by viable count on HBA plates after incubation at 22 °C and 29 °C for two days The faecal specimens used were selected among stools sent to the Bacteriological Department University Hospital Lund which were found not to contain *Salmonella* and *Shigella* spp or *Y enterocolitica* Each of the faecal specimens tested was diluted 1 100 in 10 ml PBS pH 7 4 To these suspensions 0 1 ml of one of the above mentioned *Y enterocolitica* preparations were admixed Of the infected faecal specimens 0 1 ml were used to inoculate all media tested

Recovery of Y enterocolitica *from Clinical Specimens*

One hundred faecal specimens sent to the Bacteriological Laboratory University Hospital Lund during 7 months period for the diagnosis of a possible yersiniosis were studied for *Y enterocolitica* The specimens were inoculated on DC agar and incubated at 22 °C and 29 °C for 78 hours and in enrichment buffer consisting of NaCl 8 5 g Na_2HPO_4 8 4 g KH_2PO_4 1 1 g diluted in 1 l of demineralized water The inoculated buffer was incubated at 4 °C for 3 weeks Finally the specimens were cultured on the »Y« medium which was incubated also for 2 days at 22 °C and 29 °C

In the experimental system used in this study the »Y« medium was found superior to the MC, LSU and YS media in terms of selectivity and differentiating properties for the recovery of *Y. enterocolitica* from faecal specimens. In experimentally infected

[illegible]

faecal specimens is dependent on the efficiency by which the indigenous intestinal flora, particularly *Proteus* and *Pseudomonas* spp. is suppressed. By the addition of sodium oxalate and bile salts to the »Y« medium growth of gram positive cocci was completely suppressed. By these additives the medium was also made differentiating for *Y. enterocolitica* on one hand and other gram negative rods on the other. To increase the differentiating property of the »Y«

[illegible]

distinct colour change of the medium.

Room temperature has been indicated as the optimal incubation temperature for the isolation of *Y.*

[illegible]

than 22 °C. Although larger colonies of *Y. enterocolitica* were seen on the »Y« medium when incubated at 37 °C there was a pronounced

[illegible]

dissociation did virtually not occur.

When testing pure cultures of *Y. enterocolitica* pin point size colonies of all strains tested emerged already 24 hours after incubation at 29 °C when using HBA medium. Under similar culture conditions some such strains developed visible colonies first after 48 hours of incubation when using the »Y« medium. Neither on LSU or on YS medium were any colonies detectable after incubation at 29 °C for 24 hours while on DC agar sometimes pin point colonies were found after that period of incubation. Thus in isolation studies on *Y. enterocolitica* we recommend that the »Y« medium is incubated for at least 48 hours before discarded as negative.

The »Y« medium has now been in use for approximately 2 years at the Bacteriological Laboratory, University Hospital, Lund. The experience from this period is in principle similar to that reported from the study of the series of 100 specimens described above.

The high selectivity of the »Y« medium favours the detection of *Y. enterocolitica* as compared to other media in current use for the isolation of the organism from faecal specimens. By the use of the »Y« medium the time-consuming cold enrichment can be omitted without decreasing the recovery frequency. The medium is cheap and easy to prepare.

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Table 4 *Reisolation of* Yersinia enterocolitica *on the »Y« Medium Containing either Sodium Oxalate (OXL) 1-ketoglutarate (KGL) or Sodium Succinate (SCC) (all Substances at a Concentration of 0 06 M) and on Horse Blood Agar (HBA) from 40 Experimentally Infected Faecal Specimens The Plates Were Incubated at 29 °C for 48 Hours before Read The Inoculum Used Resulted in Approximatively 30 Colony-forming Units per Plate Number of Strains Growing and Diameter of Colonies*

Medium	Number of strains growing (%)	Diameter of colonies (mm) Mean	Range
OXL	40 (100)	2 3	1 3–2 5
KGL	32 (80)	1 3	1 0–1 6
SCC	36 (90)	1 6	1 3–1 8
HBA	40 (100)	2 0	1 3–2 3

(w/v) was found to give the highest reisolation frequency and also colonies with the largest diameter However, 0 8–1 0% of sodium oxalate resulted in the highest selectivity (Table 5) In the set of experiments in which NaCl was replaced by PBS the acid produced from fermentation of lactose by gram negative bacteria caused a less distinct colour change of the indicator, viz neutral red

Comparison of the Recovery of Y enterocolitica *from Infected Stools Using Different Media*

When the »Y« medium was incubated at 29 °C for 2 days, *Y enterocolitica* could be reisolated from 99 5% of the 224 experimentally infected specimens, while when using DC, MC, LSU and YS, the bacterium could be recovered from only 90% or less of the specimens (Table 3)

Recovery of Y enterocolitica *from Clinical Specimens*

Altogether 13 strains of *Y enterocolitica* were isolated from the 100 clinical specimens of faeces studied One strain did only grow on the »Y« medium, while the 12 other strains were recovered both on DC agar and after cold enrichment

The largest colonies of *Y enterocolitica* on the »Y« medium were found when the medium was incubated at 29 °C Thus the average colony diameter after 48 hours of incubation at this temperature was 2 3 mm as compared to 2 2 and 1 9 mm at 22 °C and 37 °C, respectively

DISCUSSION

Y enterocolitica grows on simple inorganic media provided the presence of certain growth promoting factors e g thymidine Some strains of this species may also grow on minimal media containing ammonium sulphate as nitrogen source and glucose as the only source of carbon and energy (2)

Media commonly used for the isolation of *Y enterocolitica* from clinical specimens consist of peptones, or meat infusions supplemented with lactose and different agents promoting selectivity such as sodium desoxycholate, potassium tetrathionate bile salts brilliant green and cristal violet (6 17, 19)

In cultures of faecal specimens cold temperature enrichment has been used to overcome the problem of overgrowing contaminants (4, 5 12 18 19) Thus inoculation of such specimens into Rappaport broth (4 19) or phosphate buffered saline (5 12 18) followed by incubation at 4 °C for 3 weeks favour the recovery of *Y enterocolitica* from stools (12)

Table 5 *Recovery of* Yersinia enterocolitica *from Experimentally Infected Faecal Specimens after 48 Hours Incubation at 29 °C on the »Y« Medium Containing Increasing Concentrations of Sodium Oxalate Diameter of Colonies and Grading of Selectivity*

Concentration (% (w/v)) of sodium oxalate used	No of faecal specimens yielding growth (%)	Diameter of colonies (mm) Mean	Range	Degree of selectivity[a]
0 2	26 (65)	PP[b]	PP	3
0 4	29 (73)	PP	PP	3
0 6	35 (88)	1 7	1 3–1 8	2
0 8	38 (95)	2 2	1 2–2 6	1
1 0	40 (100)	2 1	1 2–2 5	1
2 0	8 (20)	PP	PP	3

[a] 1 high 2 intermediate 3 – low
[b] PP pin point colonies

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SEROLOGY OF *NEISSERIA GONORRHOEAE* CHARACTERIZATION OF RABBIT HYPERIMMUNE ANTISERA BY LINE-ROCKET IMMUNOELECTROPHORESIS FOR USE IN CO-AGGLUTINATION

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Sandstrom E & Danielsson D Serology of *Neisseria gonorrhoeae* Characterization of rabbit hyperimmune antisera by line rocket immunoelectrophoresis for the use in co-agglutination Acta path microbiol scand Sect B 88 17–26 1980

The co-agglutination (COA) and line rocket immunoelectrophoresis (L RIE) techniques were investigated for the serological classification of *Neisseria gonorrhoeae* COA with absorbed sera was found to demonstrate »strain specific« as well as cross reacting antigens The *results were dependent on the* [illegible] separate groups with regard to cross reacting antigens Based on the findings by the L RIE tests selective absorptions of antibodies were performed for use in preparation of COA reagents It was found that the reactions of these *reagents with the MOMP* reference strains could be assigned to three different antigenic classes Reagents prepared for these classes are proposed as a basis for serological classification of *Neisseria gonorrhoeae*

Key word *Neisseria gonorrhoeae* co-agglutination immunoelectrophoresis serology classification

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Serological classifications of the various bacterial genera and species such as *Salmonellae Shigellae Escherichia coli* and *Neisseria meningitidis* have greatly aided the understanding of the epidemiology and pathogenesis of infections with these organisms as well as their ecology Such classification has not yet been worked out and accepted for *Neisseria gonorrhoeae* despite serious attempts by several investigators In these studies viable or killed whole gonococcal cells were used in standard agglutination (17 19) complement fixation (14) direct or indirect immunofluorescence (IFL) (6 13 18) Other investigators used gonococcal cell fractions in various stages of purification in agar gel diffusion (6 9 10) passive haemagglutination or haemolysis (1 4 12) All the published data demonstrated the serological heterogeneity of these organisms

We recently reported the results of co-agglutination (COA) and indirect IFL in demonstrating strain specific antigens of *N gonorrhoeae* by the use of cross absorbed antibodies (8) The results correlated well with those obtained by rocket line immunoelectrophoresis with antigen-containing intermediate agar gel Our findings also indicated that

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Performance of COA tests One drop of a 1% suspension (w/v) of reagent staphylococci was mixed on a glass slide with one drop of the 1% gonococcal suspension The slide was then tilted approximately once every second for 2 min after which the reaction was read in oblique light. Negative reactions were recorded as − and positive as 1+ (weak) 2+ (moderate) and 3+ (strong and very strong)

Line rocket Immunoelectrophoresis (L RIE)

The lysates of sonicated gonococcal cells (250 mg ml⁻¹ related to the wet weight) were used in comparative studies in the L RIE tests with homologous and heterologous antigen antibody combinations arranged as shown n F

buffer in the electrophoresis vessels ionic strength 0 1 pH 8 6 was recirculated and the electrophoresis apparatus was cooled with tap water during the test runs

The area of the glass plate covered by antibody gel was about 75 cm^2 and the reference antigen line gel at the cathodic end of the plate about 10 cm^2 Each rectangular agarose area with antigen of the intermediate gel measured about 0 25 cm^2 and was separated by a corresponding rectangular agarose gel without antigen The rectangular gels were made in a mould composed of two glass plates separated by a U shaped frame 45 × 100 mm 2 mm thick 0 3 ml agarose was carefully placed at the bottom (45 mm wide) of the mould After solid fication 0 3 ml ant

sonicated

and this

tested

mixture of 2

cells against

end of the gl mm wide were poured in after which the rest of the glass plate 75 × 100 mm was covered with a mixture of 13 ml agarose and 0 3 ml (in some tests 0 6 ml) ant gonococcal antiserum

Electrophoresis was run for 22 h with a gradient of 1 5 V cm The plates were then pressed between filter papers washed twice in sal ne and once in water pressed aga n and stained with Coomassie blue as described by Axelsen et al (3)

RESULTS

1) Detection of »Strain Specific« Antigens and Cross Reacting Antigens among Gonococcal Strains by Co-agglutination Influence of Treatment of Antigens

We have showed previously that COA was a suitable method for demonstrating both »strain specific« and cross reacting antigens among gonococcal strains (8) These studies were extended in the present work with antigonococcal antibodies from ten rabbits immunized with the five S strains 14984 15057, 15000 11376 and 14190 (two rabbits for each strain) Reagent staphylococci were coated with antibodies unabsorbed or cross absorbed with the five strains Results of COA tests performed with untreated formalin fixed or boiled whole gonococcal cells are shown in Table 1

All five strains cross reacted with the unabsorbed anti GC antibodies obtained from the ten rabbits These reactions were due in part to common antigens Irrespective of the absorbing strain cross absorbed antibodies contained reactivity against heat stable antigens of the homologous strains These antigens could therefore be considered as »strain specific« antigens Cross absorbed antibodies also showed reactivity with some of the heterolog

S 15000 or S 15057 or S 11376

than CO

least reactive formalin fixed ones somewhat more reactive and boiled whole cells the most reactive The antigens responsible for these reactions could therefore be considered as cross reactive antigens which like the »strain specific« antigens seem to be heat stable

Strains that appeared to be closely related by absorption and agglutination may appear unrelated when used as immunogens This is exemplified by the anti 15000 antiserum which after absorption with strain 14190 reacted only with strain 15000 Furthermore strain 14190 also gave more reactions with the other absorbed anti 15000 antisera than any of the other absorbing strains On the other hand strain 15000 did not react with any of the absorbed antisera against strain 14190

2) Demonstration of Cross Reactive Antigens with Line rocket Immunoelectrophoresis

COA tests showed that in addition to »strain specific« antigens most anti GC antisera contained

besides »specific« antigens, there were antigens showing cross reactivity between the strains which was dependent on which particular strain was used for absorption of the anti gonococcal antibodies

In the present work the COA technique was used for further study of the serology of *N gonorrhoeae* Line rocket immunoelectrophoresis was developed to demonstrate the relationship between 16 selected gonococcal strains which could be grouped with regard to cross reacting antigens With this technique information was rapidly obtained regarding the choice of absorbing strains to be used for the preparation of defined antibodies in COA

MATERIALS AND METHODS

Neisseria gonorrhoeae *Strains*

A total of 21 strains were used in the present study Five strains S 14984 S 15057 S 15000 S 11376 and S 14190 were isolated in Stockholm in 1973 from patients with uncomplicated gonorrhoea These were selected on the basis of the results obtained from investigation of the immune response in complicated and uncomplicated gonorrhoea using the gonococcal complement fixation test (15) The 16 reference strains for the major outer membrane protein (MOMP) serotyping system (10) were kindly provided by Dr A H *Johnston* Houston Texas designated A 1 B 2 C 3 D 4 E 5 F 6 G 7 H 8 V 9 N 10 R 11 S 12 T 13 U 14 V 15 and W 16 The strains were cultured on the colony *morphology typing medium (CMT) described by Kellogg et al* (11) for 22 h in 5% CO_2 at 37 °C unless otherwise stated The MOMP reference strains were received unselected as regards colony morphology type T2 colonies with golden brown of dark brown colour were obtained from strains D 4 F 6 H 8 R 11 V 15 and T 13 while T1 like colonies of varying colour (light golden brown) had to be accepted for A 1 C 3 E 5 N 10 S 12 and U 14 T3 like for B 2 and G 7 and T5 like for V 9 (3) They were selected and recultured daily over a two week peri d bef re freezing at −70 °C in narrow tubes fr m w h h so all samples could be thawed when needed (8) Gol l r wn or dark brown colonies of the various types were preferred for use in the subsequent experiments

Production of Antisera

Preparation of whole cell antigens Organisms of T2 colony morphology types of the five S strains were cultured on CMT medium and seeded on agar plates

7 4 and treated with ...
previously (5) The organisms were suspended in saline to a concentration corresponding to McFarland No 4 and used for immunization within two weeks

Preparation of sonicated antigens Organisms of the 16 gonococcal MOMP serotypes *A 1 to W 16 (10)* we cultured on CMT medium in the same way as the col morphology types described above The organisms we harvested in PBS pH 7 4 centrifuged for 15 min a 2 500 × *g* and then adjusted to a concentration of 2 *mg ml*$^{-1}$ *in PBS The cells were disrupted by* sonica whith a 100 watt MSE ultrasonic apparatus as describe previously (8) Sonicated cells prepared in this way we used for absorption of antiserum as described below Fo immunization of rabbits the sediment was obtained b centrifuging the lysate at 30 000 × *g* (Beckmen ultracentrifuge) for 20 min washing once in sal ne, an *finally suspending in saline to 25 times the orig* volume of the lysate The sediment was stored in sm aliquots at −70 °C until use

Immunization White female rabbits weighing appr imately 2 5 kg were used Two rabbits were immun with the formalin fixed whole cells for each of the five S strains with an initial intramuscular injection of 2 m *antigen mixed with 2 ml Freund s complete* adjuva Two weeks later the animals were given a series of in *injections viz 0 1 ml 0 25 ml 0 5 ml and 1 0 ml of the* same antigen at intervals of 3–4 days The series wa repeated after two weeks and the animals were *bled two* weeks after the last injection Animals immunized w the sediment of the 16 gonococcal MOMP serotypes of Johnston et al (see above) were given a total of f u subcutaneous injections at intervals of two weeks ea *time with 2 ml antigen mixed with 2 ml* Freund's complete adjuvant The animals were bled two wee after the fourth injection

Absorption of Antiserum

Absorption was preformed by mixing one volume of antiserum with four volumes of the sonicated cells (se *above) and incubating for 1 h in water bath at 37 °C a* then overnight at + 4 °C The absorbed antiserum which in this way was *diluted 1 5 was recovered b* centrifugation at 30 000 × *g* for 20 min and if not us the same day stored at −70 °C until use On som occasions undiluted antisera were absorbed with th corresponding volume of packed formalin fixed cells f 4 h at 37 °C and overnight at + 4 °C The antiserum wa recovered by centrifugation and if residual activity to th absorbing strains persisted the absorption was repeate two or three times with heat treated cells for the sam *time and with the same concentrations*

C agglutination (COA)

Antigens The gonococcal strains were grown on CM medium The cells were harvested in PBS pH 7 4 an used as untreated whole cells (UWC) with the addition o 0 1% NaN_3 (w/v) as formalin fixed whole cells (FWC (5) or as heated whole cells (HWC) after boiling for 1 in a water bath The gonococcal suspensions wer adjusted to a 1% concentration (w/v)

Preparation of reagents The production of protein A *staphylococci reagent staphylococci sensitized* wit unabsorbed or absorbed antigonococcal antibodies and *control reagents followed the* procedure described previously (8)

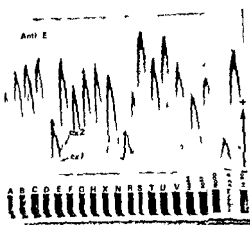


Fig 1 Line rocket immunoelectrophoresis with anti E 5 in the reference antibody gel sonicated cells of strain E 5 in the line gel and sonicated cells of homologous and heterologous strains in the intermediate gel

cx 1 and *cx 2* designate two different precipitin lines parallel to the line gel connecting rockets formed over the antigens in the intermediate gel (type 1)

Capital letters refer to the MOMP reference strains

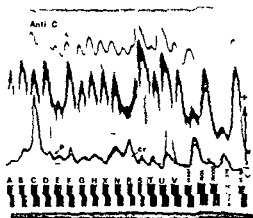


Fig 2 Line rocket immunoelectrophoresis with anti C 3 in the reference antibody gel sonicated cells of strain C 3 in the line gel and sonicated cells of homologous and heterologous strains in the intermediate gel

pr designates partial reaction of identity between the precipitin line parallel to the line gel and the rocket (type 1 reaction)

cr designates rocket over antigen in intermediate gel not connected to a visible line parallel to the line gel (type 2 reaction)

Capital letters refer to the MOMP reference strains

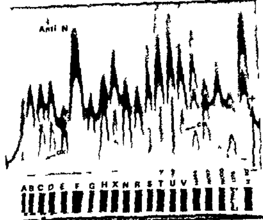


Fig 3 Line rocket immunoelectorphoresis with anti N 10 in the reference antibody gel sonicated cells of strain C 3 in the line gel and sonicated cells of homologous and heterologous strains in the intermediate gel

cx designates precipitin line parallel to the line gel connecting rockets over antigen in the intermediate gel (type 1 reaction)

cr designates rocket over antigen in the intermediate gel not connected to a visible line parallel to the line gel (type 2 reaction)

Capital letters refer to the MOMP reference strains

were at times seen as partial reactions (pr Fig 2) In the *type 2* reaction a rocket line formed by the antigens of the intermediate gel could be identified but no line of identity connected these rockets with the concentrations of antiserum used and antigen in the line gel (cr Fig 2) These precipitin lines represented another antigen or antigens related to the homologous strain In the *type 3* reaction inverted or no rockets formed over the antigens in the intermediate antigen gel These reactions (designated −) indicated lack of specific or cross reacting antigens In the *type 4* reaction antigens in the intermediate agar gel formed rocket precipitin lines with reactions of identity at the anodic end of the agarose (Fig 1 and 2) for all the 16 MOMP strains These rockets represented the common antigens Examples of two independent *type 1* reactions are shown in Fig 1 one with strains B 2 D 4 E 5 G 7 R 11 and V 15 (cx1) and the second with strains B 2 E 5 G 7 H 8 N 10 and V 15 (cx2) in the test with the E 5 anti E 5 combination The C-3 anti C-3 combination (Fig 2) illustrates *type 1* reaction for strains B 2 C-3 E 5 F 6 G 7 H 8 N 10 and R 11 Partial identity can be seen in Fig 2 for strains D 5 and V 15 *Type 2* reactions were obtained with strains C 3 D-4 R 11 and V 15

TABLE 1 *Co-agglutinations of GC Cells with Reagent Staphylococci Coated with Anti-GC Antibodies Absorbed w* … *Homologous or Heterologous* N gonorrhoeae *Organisms Reactions Graded as Described in Material and Methods*

Antiserum against strain (rabbit)[a]	Absorbing strain	COA reactions with untreated formalin treated and heated whole cells of *Neisseria gonorrhoeae*														
		S 14984			S 15057			S-15000			S 11376			S-H X	
		UWC[b]	FWC[c]	HWC[d]	UWC	FWC	HWC	UWC	FWC	HWC	UWC	FWC	HWC	UWC	FWC
S 14984	–	3+	3+	3+	3+	3+	3+	3+	3+	3+	3+	3+	3+	3+	3+
(b_1/b_2)	S 15000	3+	3+	3+	–/2+	–/2+	2+	–	–	2+/–	–/2+	+/3+	3+	2+	+
	S 15057	3+	3+	3+	–	–	2+/–	–	–	–	–	+	3+/+	2+/–	+
	S 11376	2+	3+	3+	–	–	2+/–	–	+	2+/–	–	–	2+/–	–	–
	S 14190	2+	2+	2+	–	–	–	–	–	–	–	–	+	–	
	S 14984	–	–	–	–	–	–	–	–	–	–	–	–	–	–
S 15057	–	3+	3+	3+	3+	3+	3+	3+	3+	3+	3+	3+	3+	3+	3+
(d_1/d_2)	S 14190	+	2+/–	3+/–	3+	3+	3+	–	2+/–	3+/+	+	3+/–	3+/+	–	–
	S 15000	–	–	3+/–	3+	3+	3+	–	–	+	–	+	3+/–	+	–
	S 14984	–	–	3+/–	3+	3+	3+	–	+	3+/+	–	+	3+/–	–	–
	S 11376	+	–	–	3+	3+	3+	–	–	2+/–	–	–	–	–	–
	S 15057	–	–	–	–	–	–	–	–	–	–	–	–	–	–
S 15000	–	3+	3+	3+	3+	3+	3+	3+	3+	3+	3+	3+	3+	3+	3+
(f_1/f_2)	S 14984	–	–	–	–/2+	–/2+	2+	3+	3+	3+	–	–/2+	–/2+	–/2+	/?
	S 15057	–	–	+	–	–	–	3+	3+	3+	–	–	–/3+	+	+
	S 11376	–	–	–	–	–	–/2+	3+	3+	+/3+	–	–	–	–	–/?
	S 14190	–	–	–	–	–	–	3+	3+	+/3+	–	–	+	–	–
	S 15000	–	–	–	–	–	–	–	–	–	–	–	–	–	–
S 11376	–	3+	3+	3+	3+	3+	3+	3+	3+	3+	3+	3+	3+	3+	3+
(c_1/c_2)	S 14190	+	+	2+	–/2+	2+	+	–	–	–	2+	2+	3+	–	–
	S 15000	+	+	3+	+	+	+	–	–	–	2+	2+	3+	–	–
	S 15057	+	+	3+	–	–	–	–		–	2+	2+	3+/+	–	–
	S 14984	–	–	–	+	–	–	–	–	–	2+	2+	2+	–	
	S 11376	–	–	–	–	–	–	–	–	–	–	–	–	–	–
S 14190	–	3+	3+	3+	3+	3+	3+	3+	3+	3+	3+	3+	3+	3+	3+
(e_1/e_2)	S 15057	–/2+	2+	3+	–	–	–	–	–	+	–	–	2+/–	3+	3+
	S 15000	–/2+	+	3+	–	–	–	–	–	–	–	–	2+/–	2+	1+
	S 14984	–	–	–	–	–	–	–	–	–	–	–	–	3+	2+
	S 11376	–	–	+	–	–	–			–		–	–	2+/–	3+
	S 14190	–	–	–	–	–	–			–	–		–	–	–

a) COA reactions of the individual rabbit presented only when there was a difference of more than 1 +
b) UWC – untreated whole cells c) FWC = formalin treated whole cells d) HWC – heated (100 °C) whole cells

antibodies to more than one cross-reactive antigen, thus resulting in complex patterns To cover all combinations with 10 strains and their corresponding antisera, 100 absorptions and 1000 tests would be required if the relationship was unknown However, we have shown in a previous work that the results of COA tests coincided well with those obtained by rocket line immunoelectrophoresis (8) In the present work, we therefore developed the line-rocket immunoelectrophoresis described in Materials and Methods, with the aim of guiding and reducing the number of absorptions for anti GC antibodies for use in COA tests We decided to use hyperimmune rabbit antisera against the 16 gonococcal serotypes described by *Johnston et al* (10) known to differ in at least one antigen Antisera, one for each strain, that gave a precipitation pattern typical of previously described strain-specific antigens (8), were selected for these studies

In these L-RIE tests, antigen preparations from more than 20 GC strains could be compared with a reference antigen-antibody system and four types of reactions could be observed These are referred to as *type 1 type 2 type 3* and *type 4* reactions In the *type 1* reaction, a line of identity connected the precipitin line of the reference antigen line gel with the rocket(s) formed by the antigens of the homologous and one or more of the heterologous strains in the intermediate gel These precipitin lines thus represent cross-reacting (cx, Fig 1) antigen complexes between the homologous and the heterologous strains One or two such cross-reacting antigen-antibody systems could be detected in a particular test run Dissociations of the

TABLE 3 *Co agglutination Reactions of Heated (100 °C 1 h) whole Gonococcal Cells from the MOMP Reference Strains with Reagent Staphylococci Coated with Anti GC Antibodies Absorbed with Homologous and Heterologous Strains Reactions Graded as Described in Material and Methods*

itiserum ainst ain bbit)	Absorbing strain	Results of COA tests with MOMP reference strains															
		W	B	G	E	R	D	V	N	H	C	X	S	T	U	A	F
5 S 32)	B 2	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	G 7	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	E 5	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	R 11	–	2+	3+	3+	–	–	+	+	+	–	–	–	–	–	–	–
	D 4	–	2+	2+	+	+	–	–	–	+	–	–	–	–	–	–	–
	V 15	–	2+	3+	3+	+	+	–	+	–	–	–	–	–	–	–	–
	N 10	–	3+	3+	3+	3+	+	2+	–	–	–	–	–	–	–	–	–
	H 8	–	3+	3+	3+	3+	+	2+	–	–	–	–	–	–	–	–	–
	A 1	–	3+	2+	3+	3+	+	2+	3+	3+	–	–	–	–	–	–	–
4 S31)	W 16	–	3+	2+	3+	3+	2+	3+	2+	2+	3+	–	–	–	–	+	2+
	B 2	–	–	–	–	3+	3+	3+	–	–	3+	–	–	–	–	–	–
	G 7	+	+	–	2+	2+	3+	3+	–	–	3+	–	–	–	–	–	–
	E 5	–	–	–	–	+	3+	2+	–	–	–	–	–	–	–	–	–
	R 11	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	D 4	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	V 15	–	–	–	–	+	+	–	–	–	–	–	–	–	–	–	–
	C 3	2+	+	+	+	3+	3+	3+	–	–	–	–	–	–	–	–	–
	A 1	–	2+	–	–	3+	3+	3+	–	–	2+	–	–	–	–	–	–
	F 6	nd[a]	3+	2+	3+	3+	3+	3+	+	+	3+	–	–	–	2+	–	–
	C 3 + B 2	–	–	–	–	–	3+	3+	–	–	–	–	–	–	–	–	–
10 S 37)	B 2	nd	–	–	–	+	3+	–	3+	3+	3+	3+	3+	–	–	2+	2+
	G 7	nd	–	–	2+	+	3+	+	3+	3+	3+	3+	2+	–	–	2+	2+
	R 11	–	–	–	+	–	3+	–	3+	2+	+	3+	–	–	–	2+	–
	D 4	–	–	–	–	–	–	–	3+	3+	3+	3+	3+	–	–	–	–
	V 15	–	–	–	–	–	–	–	3+	3+	3+	3+	3+	–	+	–	–
	N 10	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	H 8	–	–	–	–	+	–	–	3+	–	–	–	–	–	–	–	–
	C 3	–	–	–	+	–	+	–	2+	+	–	2+	–	–	–	+	–
	V 9	–	+	+	+	2+	2+	–	2+	2+	–	–	–	–	–	+	+
	S 12	–	+	–	2+	–	+	+	3+	3+	–	3+	–	–	–	2+	–
	U 14	–	–	–	2+	–	3+	–	3+	2+	+	2+	–	–	–	2+	–
12 S 39)	W 16	–	–	–	–	–	–	–	3+	3+	3+	3+	3+	3+	3+	2+	–
	B 2	2+	–	–	+	2+	3+	–	3+	3+	3+	3+	3+	3+	3+	3+	3+
	R 11	–	–	–	–	–	–	–	3+	2+	2+	3+	3+	3+	3+	–	–
	N 10	–	–	–	–	–	–	–	–	–	–	–	2+	–	–	–	–
	H 8	2+	–	–	–	–	–	–	–	–	–	–	3+	–	–	–	–
	C 3	2+	–	–	–	–	–	–	–	+	–	+	2+	–	2+	–	–
	V 9	–	–	–	–	–	–	–	–	+	–	–	3+	–	–	–	–
	S 12	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	T 13	–	–	–	–	–	–	–	–	+	–	–	2+	–	–	–	–
	U 14	–	–	–	–	–	–	–	–	–	–	–	3+	–	–	–	–
	A 1	–	–	–	–	–	–	–	3+	3+	3+	3+	3+	3+	3+	–	–

[a] nd not done

TABLE 2 *Results with Line-rocket Immunoelectrophoresis Using Antisera to the MOMP Reference Strains Tes against Sonicated Cells of the Homologous and Heterologous Strains in the Intermediate Gel*

Antiserum against strain (rabbit)	Antigen in line gel	L-RIE reactions with antigens from the MOMP reference strains in the intermedia															
		W	B	G	E	R	D	V	N	H	C	X	S	T	U	A	
B-2	B-2	–[a]	cx[b]	cx	cx	cx	cx	pr[c]	cx	cx	–	–	–	–	–	–	
(KS 29)		–	cr[d]	cr	cr	–	–	–	–	–	–	–	–	–	–	–	
G-7	G-7	–	cx	cx	cx	cx	–	–	cx	cx	–	–	–	–	–	–	
(KS 34)		cr	–	cr	–	–	–	–	–	–	–	–	–	–	–	–	
E-5	E-5	–	cx1	cx1	cx1	cx1	cx1	cx1	–	–	–	–	–	–	–	–	
(KS 32)		–	cx2	cx2	cx2	–	–	cx2	cx2	cx2	–	–	–	–	–	–	
		–	–	–	cr	cr	–	–	–	–	–	–	–	–	–	–	
R-11	R-11	nd[e]	cx1	cx1	cx1	cx1	cx1	cx1	cx1	cx1	cx1	–	–	–	–	–	
(KS38)		nd	–	–	–	cx2	cx2	cx2	–	–	cx2	–	–	–	–	–	
D 4	D 4	nd	–	–	–	–	cx	cx	–	–	–	–	–	–	–	–	
(KS 31)		nd	cr	cr	cr	cr	cr	cr	–	–	cr	–	–	–	–	–	
V-15	V-15	nd	–	cx	cx	cx	cx	cx	cx	cx	cx	–	–	–	–	–	c
(KS 42)		nd	cr	cr	cr	–	cr	cr	–	–	cr	–	–	–	–	–	
N-10	N-10	nd	–	–	–	–	–	–	cx	cx	cx	cx	cx	–	–	–	
(KS 37)		nd	–	–	cr	–	–	–	cr	cr	–	–	–	–	–	–	
H-8	H 8	–	cx	cx	cx	–	–	–	cx	cx	–	–	–	–	–	–	
(KS 44)		–	–	–	–	–	–	–	–	–	–	cr	–	–	–	–	
C-3	C-3	–	cx	cx	cx	cx	pr	pr	cx	cx	cx	–	–	–	–	–	c
(KS 30)		–	–	–	–	cr	cr	cr	–	–	cr	–	–	–	–	–	
X-9	X-9	–	–	–	–	–	–	–	–	–	–	cx	–	cx	cx	–	
(KS 36)		–	cx	cx	cx	–	–	–	–	–	cx	cx	–	cx	cx	–	
		–	–	–	cr	cr	–	–	cr	–	cr	–	–	–	–	–	
S-12	S 12	–	–	–	–	–	–	–	cx1	cx1	–	–	cx1	–	–	–	
(KS 39)		–	–	–	–	–	–	–	cx2	cx2	cx2	–	cx2	–	–	–	
		–	–	–	–	–	–	–	cr	–	–	cr	–	cr	cr	cr	cr
T-13	T-13	–	–	–	–	–	–	–	–	–	–	–	–	cx1	cx1	–	-
(KS 40)		–	–	–	–	–	–	–	cx2	–	cx2	–	–	cx2	cx2	–	-
		–	–	–	–	–	–	–	cx3	–	–	–	–	cx3	cx3	–	cx
U-14	U 14	nd	–	–	cx	–	–	cx	cx	cx	cx	cx	–	cx	cx	cx	cx
(KS 41)		nd	–	–	–	–	–	–	–	–	–	–	–	cr	cr	–	-
A-1	A 1	–	–	–	–	–	–	–	–	cx	–	–	–	–	–	cx	cx
(KS 28)		–	–	–	–	–	–	–	–	–	–	cr	–	–	–	–	-
F-6	F 6	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	cx
(KS 43)																	

[a] – = type 3 reaction no rocket formed over the antigen in the intermediate gel
[b] cx = type 1 reaction see text and Fig 1
[c] pr = partial reaction (cf Fig 2)
[d] cr = type 2 reaction (cf Fig 2)

TABLE 3 *Co agglutination Reactions of Heated (100 °C 1 h) whole Gonococcal Cells from the MOMP Reference Strains with Reagent Staphylococci Coated with Anti GC Antibodies Absorbed with Homologous and Heterologous Strains Reactions Graded as Described in Material and Methods*

itiserum ainst ain bbit)	Absorbing strain	Results of COA tests with MOMP reference strains															
		W	B	G	E	R	D	V	N	H	C	X	S	T	U	A	F
5 S 32)	B 2	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	G 7	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	E 5	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	R 11	–	2+	3+	3+	–	–	+	+	+	–	–	–	–	–	–	–
	D 4	–	2+	2+	+	+	–	–	–	+	–	–	–	–	–	–	–
	V 15	–	2+	3+	3+	+	+	–	+	–	–	–	–	–	–	–	–
	N 10	–	3+	3+	3+	3+	+	2+	–	–	–	–	–	–	–	–	–
	H 8	–	3+	3+	3+	3+	+	2+	–	–	–	–	–	–	–	–	–
	A 1	–	3+	2+	3+	3+	+	2+	3+	3+	–	–	–	–	–	–	–
4 S31)	W 16	–	3+	2+	3+	3+	2+	3+	2+	2+	3+	–	–	–	–	+	2+
	B 2	–	–	–	–	3+	3+	3+	–	–	3+	–	–	–	–	–	–
	G 7	+	+	–	2+	2+	3+	3+	–	–	3+	–	–	–	–	–	–
	E 5	–	–	–	–	+	3+	2+	–	–	–	–	–	–	–	–	–
	R 11	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	D 4	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	V 15	–	–	–	–	+	+	–	–	–	–	–	–	–	–	–	–
	C 3	2+	+	+	+	3+	3+	3+	–	–	–	–	–	–	–	–	–
	A 1	–	2+	–	–	3+	3+	3+	–	–	2+	–	–	–	–	–	–
	F 6	nd[a]	3+	2+	3+	3+	3+	3+	+	+	3+	–	–	–	2+	–	–
	C 3 + B 2	–	–	–	–	–	3+	3+	–	–	–	–	–	–	–	–	–
10 \S 37)	B 2	nd	–	–	–	+	3+	–	3+	3+	3+	3+	3+	–	–	2+	2+
	G 7	nd	–	–	2+	+	3+	+	3+	3+	3+	3+	2+	–	–	2+	2+
	R 11	–	–	–	+	–	3+	–	3+	2+	+	3+	–	–	–	2+	–
	D 4	–	–	–	–	–	–	–	3+	3+	3+	3+	3+	–	–	–	–
	V 15	–	–	–	–	–	–	–	3+	3+	3+	3+	3+	–	+	–	–
	N 10	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	H 8	–	–	–	–	+	–	–	3+	–	–	–	–	–	–	–	–
	C 3	–	–	–	+	–	+	–	2+	+	–	2+	–	–	–	+	–
	X 9	–	+	+	+	2+	2+	–	2+	2+	–	–	–	–	–	+	+
	S-12	–	+	–	2+	–	+	+	3+	3+	–	3+	–	–	–	2+	–
	U 14	–	–	–	2+	–	3+	–	3+	2+	+	2+	–	–	–	2+	–
-12 KS 39)	W 16	–	–	–	–	–	–	–	3+	3+	3+	3+	3+	3+	3+	2+	–
	B 2	2+	–	–	+	2+	3+	–	3+	3+	3+	3+	3+	3+	3+	3+	3+
	R 11	–	–	–	–	–	–	–	3+	2+	2+	3+	3+	3+	3+	–	–
	N 10	–	–	–	–	–	–	–	–	–	–	–	2+	–	–	–	–
	H 8	2+	–	–	–	–	–	–	–	–	–	–	3+	–	–	–	–
	C 3	2+	–	–	–	–	–	–	–	+	–	+	2+	–	2+	–	–
	X 9	–	–	–	–	–	–	–	–	+	–	–	3+	–	–	–	–
	S 12	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	T 13	–	–	–	–	–	–	–	–	+	–	–	2+	–	–	–	–
	U 14	–	–	–	–	–	–	–	–	–	–	–	3+	–	–	–	–
	A 1	–	–	–	–	–	–	–	3+	3+	3+	3+	3+	3+	3+	–	–

[a] nd not done

On the basis of these findings, the strains could be arranged according to *type 1*, *type 2* and *type 3* reactions (Table 2) The strains could be placed in an apparantly continuous pattern due to covarying cross-reacting antigens revealed by *type 1* reactions In this way the MOMP strains could be divided into two main groups The first consisted of strains B-2, G-7, E-5, R-11, D 4 and V-15, which with their corresponding antibodies formed one or two *type 1* reactions with three to six of these strains but not with strains S-12, T-13, U-14 or A-1 These strains were referred to the second group, together with strains N-10, H-8, C-3 and X-9 which gave *type 1* reactions in various combinations within both groups Strains S-12, T-13, A-1 and F-6 gave only *type 1* reactions within the second group *Type 2* reactions behaved similarly By using heterologous antigens in the reference line gel (strain C-3 of the second group) (Fig 3), *type 1* reactions were confined to the second group and *type 2* reactions in various combinations within the first and second groups

3) *Correlation between L-RIE and COA*

Each antiserum used in L RIE tests (Table 2) was absorbed separately with three different strains one heterologous strain within the same group, one cross-reacting and one non reacting strain of the other group Reagent staphylococci coated with these antibodies were then used to test heated (100 °C) whole cells of the 16 MOMP reference strains The reaction patterns obtained could be referred to three classes The first consisted of absorbed antibodies against antigens common to strains within the same group (see L-RIE results in Table 2) as the immunizing strain the second of absorbed antibodies against the immunizing strain (sometimes also against another closely related strain within the same group) and the third of absorbed antibodies against cross reacting antigens of strains belonging to both groups These results are shown in Table 3 with four selected antisera that were absorbed with the strains which gave *type 1* and/or *type 2* reactions and with a few which gave *type 3* reaction in the L-RIE

Anti E-5 Absorption of this antiserum with strains B 2 G 7 and E 5 showed that these strains shared most antigens corresponding to the *type 1* reactions with anti E-5 in L RIE (Table 2) Cross reactive antigens were found in strains R-11 D-4 and V-15 as well as N 10 and H 8 when anti E 5 was absorbed with a strain belonging to the second group in the L RIE tests again indicating two covarying cross-reacting antigens one belonging to class 1, the other to class 3

Anti-D-4 The COA tests with absorbed anti-D 4 showed that strains R-11, D 4 and V-15 shared at least two antigens This could be shown by absorbing with strain B-2, after which reactions were obtained with R-11, D 4, V-15 of the same group and C-3 of the other group (class 3) After absorption with C-3, reactions were obtained with W-16, B 2, G-7, E-5, R-11, D 4 and V-15 (class 1) Only strains D-4 and V-15 reacted in the COA test after absorption with B-2 and C-3 (class 2) which corresponds to the *type 1* reaction in the L RIE *Reactions corresponding to classes 1 and 3* appeared as *type 2* reactions in L-RIE It was also clear that the results of the COA tests were dependent on the strain used for absorption

Anti-N-10 After absorption of this antiserum with strains D-4 or V-15, strong reactions were obtained with strains N-10, H 8 C-3 X 9 and S-12, which indicated that these strains shared the same antigens (class 1) This corresponded to *type 1* reactions in L-RIE An almost monospecific antiserum to strain N-10 was obtained by absorption with H-8, which was closely related to N-10 according to L-RIE tests After absorption with unrelated strains (for example U-14) numerous and often weak cross reactions were observed

Anti-S-12 A strain specific antigen of S-12 (class 2) was demonstrated after absorption with N 10 or U-14 The strong reactions with strains N 10 H 8 C-3 X 9 S 12 T 13 and U 14 after absorption with R-11 or A-2 correspond to antigens of the same group and can therefore be referred to class 1

DISCUSSION

Most attempts to achieve serological classification of *Neisseria gonorrhoeae* have suffered from the assumption that a single major antigen exists in all gonococci which can vary only to a limited extent Notable exceptions are the investigations by *Wilson* who from agglutination studies of whole organisms defined the antigenic pattern of a particular strain by a formula (19) and those by *Maeland* (12) who from studies of antigenic endotoxin fractions described the occurrence of varying numbers of antigenic factors in a particular strain Using isolated outer membranes for serotyping gonococci by the Ouchterlony method *Johnston et al* (10) pointed out that even these purified antigens gave unwanted and disturbing antibodies if the immunization was not carefully supervised

In the present work we showed by both co agglutination and immunoelectrophoresis techniques that rabbit hyperimmune anti gonococcal antisera contained reactivity against common as

ell as »strain-specific« and cross reactive antigens hese findings are in agreement with those of a revious report (8) and they further support the bservations by others that the antigenic pattern of a articular gonococcal strain could best be described y a formula (12 19)

The COA method which is not disturbed by the nherent auto agglutinating properties of many GC trains was found suitable for detecting various intigens However the total antigenic pattern of a particular strain was not revealed until the antibo lies were extensively cross absorbed and cross ested with homologous and heterologous strains procedures that are laborious and time consuming The results in Table 1 also show that the patterns obtained were complex and dependent on the immunizing absorbing and agglutinating properties of a particular strain They were also to a minor extent dependent on which individual rabbit immune serum was used It was noteworthy that whole cells heated at 100 °C were more reactive than those untreated or formalin fixed We have shown previously that for example untreated cells from T4 colony morphology types might even be inagglutinable though not after heat treatment (8) Untrea ed organ sms that do not react in a COA test should therefore also be tested after such treatment

We showed previously that there was excellent agreement between results obtained with the COA method with cross absorbed antibodies and crossed and rocket line immunoelectrophoresis with anti gen conta n ng n ermed

when using a homologous antigen antibody system permitted comparison of the antigenic relationship between more than 20 strains This is the advantage of L RIE as compared to rocket line and crossed immunoelectrophoresis

The L RIE was applied to the 16 MOMP reference strains (10) which then could be divided into two main groups Strains B 2 G 7 E 5 R 11 D-4 and V 15 were clearly separated from strains S 12 T 13 U 14 and A 1 Strains N 10 H 8 C 3 and X 9 reacted with antisera against both of these sets of strains and their corresponding antisera also reacted with both sets The observed reaction patterns with strains B 2 G 7 E 5 N 10 H 8 and with strains R 11 D-4 V 15 C 3 led to the

R 11 D-4 and V 15 belonging to the first antigenic group The second antigenic group of class 1 was represented by the rest of the MOMP strains Class 3 consisted of rains in both these groups This concept was confirmed when absorptions of antisera for the COA test were carried out with three different strains i e. absorption with a strains within the same antigenic group absorption with a cross reacting strain and absorption with a non reacting strain respectively from the other antigenic group Absorbed antibodies could then be produced with a reaction pattern in COA tests distinctive for class 1

It was also found that closely related strains often absorbed all activity only occasionally leaving »specific« antisera against 1 or 2 MOMP reference strains Antigens responsible for these COA reactions could then be referred to a third class Antibodies absorbed with unrelated strains were often not suitable for analysis in COA tests because of multiple weak and complex reaction patterns thus contrasting with the strong reactions of class specific antisera absorbed with more closely related strains

The procedures described have been found useful and reliable for the serological classification of gonococcal strains described in recent serological studies by other authors and also for the classification of β lactamase producing strains and strains from patients with disseminated gonococcal infection (16)

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SEROLOGY OF *NEISSERIA GONORRHOEAE* CLASSIFICATION BY CO-AGGLUTINATION

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The co-agglutination (COA) method has been adapted for serological classification of *Neisseria gonorrhoeae* COA reagents were prepared with selectively absorbed rabbit hyperimmune antibodies against gonoccal (GC) major outer membrane protein (MOMP) serotype strains Using these reagents the 16 MOMP reference strains could be referred to at least three antigen classes tentatively named W J and M The GC antigens of class W were divided into three groups I II and III and they were in part sensitive to pronase The antigens of class J reflected strain specific or serotype reactions some sensitive and others resistant to proteolytic enzymes The antigens of class M were sensitive to periodate and resistant to pronase Strains used in serological studies by other authors were tested The properties of class W correlated well with those of the so-called micro immunofluorescence and immunotype systems and class M with those of the so-called endotoxin and acid polysaccharide systems Strains from three different laboratories could all be grouped by class W and M reagents Identical strains obtained independently from different laboratories gave very similar reaction patterns with the reagents available Repeated GC-isolates from patients infected with beta lactamase producing strains showed stable reactions with class W and J reagents while there was a time related variation of the class M pattern We have found that the COA method is rapid easy and reproducible in the serological classification of *Neisseria gonorrhoeae* and all the 117 GC-strains tested could be classified

Key words *Neisseria gonorrhoeae* co agglutination serology classification

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Ever since the beginning of this century many tempts have been made to achieve a serological assification of *Neisseria gonorrhoeae* The older orks were summarized by *Reyn* (19) and *Wilson* [illegible] was able to overcome most of the difficulties in irect agglutination of gonococci. With absorbed ntisera against undefined antigens he was able to haracterize gonoccal strains by a formula Further tudies along these lines were however discoura ged by the finding that gonococci could change serologically on prolonged subculture *Maeland* (17) working with indirect haemagglutination (HA) with periodate sensitive but pronase stable antigens prepared from the endotoxin found antigenic factors of three reference strains best represented by a formula *Apicella* (2) purified an acid polysaccha ride antigen for use in agar gel diffusion (AGD) and HA studies Using absorbed antisera he was able to define five serotypes (3) *Johnston et al* (14) studied by AGD an antigen characterized by major outer membrane protein (MOMP) in polyacrylamide gel electrophoresis and found 16 serotypes in a broad panel of strains *Wang et al* (22) applied the so called micro-immunofluorescence (Micro-IF) to undefined gonococcal antigens with absorbed mouse antisera. With this system three groups with subgroups were observed and a correlation with

auxotypes and clinical manifestations was demonstrated

We have previously explored the potential use of the co agglutination (COA) method for serological studies of gonococci and the results were strongly correlated to those obtained by indirect immunofluorescence and immunoelectrophoresis (8) Based on the findings by the so-called line rocket immunoelectrophoresis (L RIE) defined reagents could be prepared for COA by selected absorptions When applied to the 16 MOMP reference strains described by *Johnston et al* (14) the results of COA tests with these absorbed antibodies could be referred to at least three classes of antigens We suggested that this might form the basis of a serological classification of gonococci according to a multifactor model

These studies were extended in the present work, and absorbed antibodies were prepared against the MOMP reference strains to identify the three classes of antigens by COA tests These reagents were then used to classify strains used in serological studies by *Maeland* (17) *Geizer* (12) *Apicella* (3) *Arko et al* (4) and *Wang et al* (22) as well as other strains kept at our and other laboratories Included were also β lactamase producing gonococcal strains isolated from six patients

MATERIALS AND METHODS

Neisseria gonorrhoeae *Strains*

One hundred and seventeen gonococcal (GC) were included in the present study They were ted by six groups of reference strains in all 45 and three groups of other defined strains in all 26 designations and sources of these strains are shown Table 1 Forty six strains were isolated from six pa infected with β lactamase producing GC

Isolates HCA 1–21 were obtained from a male pa (HCA) with multiple sexual exposures in the Far Ea They were isolated from urethral and pharyng specimens over a 73-day period due to treatm failures Isolates HCA 1–2 were from urethra the from the pharynx All isolates except HCA 2 β lactamase

Isolates BA 1–8 all β lactamase producers obtained from a male patient (BA) with multiple se exposures in the Far East They were isolated over a day period BA 1 from the urethra the rest f pharynx

Isolates RC 1–3 all β lactamase producers were fr a male patient (RC) with a single sexual exposure in th Far East The strains were isolated from ureth specimens over a 6-day period

Isolate HA 1 was from the urethra of a male pa (HA) with a history of sexual intercourses in Poland

Isolate TB 1 was from a male patient (TB) with fu

TABLE 1 *Compilation of* Neisseria gonorrhoeae *Strains Used in the Present Study*

A Reference Strains Used by Others in Serological Studies

1) *Major Outer Membrane Protein (MOMP) Strains* (14) 16 strains obtained from Dr *K H Johnston* Hou Texas USA Designations see Table 2
2) *Micro Immuno fluorescence (Micro IF) Reference Strains* (22) eight strains obtained from Drs *K K Ho* and *J Knapp* Seattle Washington USA Designations see Table 3
3) *Immunotyping Reference Strains* (4) eight strains obtained from Dr *K H Wong* Atlanta Georgia U Designations see Table 3
4) *Endotoxin Reference Strains* (17) three strains obtained from Dr *J A Maeland* Trondheim Norw Designations see Table 3
5) *Acid Polysaccharide Serogroup Reference Strains* (3) five strains obtained from Dr *M A Apicella* Buff N Y USA Designations see Table 3
6) *Gel Diffusion Reference Strains* (12) five strains obtained from Dr *I Geizer* Prague Czechoslovak Designations see Table 3

B Other Defined Strains

1) *Seattl Stra ns* nine in all six obtained from Drs *K K Holmes* and *J Knapp* three from Dr *T Bucha an* Seattle Washington USA Designations see Table 4
2) *Örebr Str s* six in all Four strains described in a previous work (8) one strain 732 from a pati with disseminated gonococcal infection (DGI) one strain P9 obtained from Dr *M E Ward* Southampt England Designations of the other four strains see Table 4
3) *Stockh l n Strains* 11 in all Five were described in a previous work (21) designations see Table 4 Str 23892 isolated from the knee joint and strains He Ge De and Bj from urogenital sepcimens of five fema with DGI Strain K1 was isolated from the pharynx of a female with a history of arthralgia two months af intercourse

C Strains from Patient Infected with *β – Lactamase Producing Strains*

Forty six isolates f om six patients infected with β – lactamase producing GC were incl ded n the serolog tests H [illegible]

l exposures in the Far East Urethral discharge ed four days after his return to Stockholm and l exposure to patient UL (see below) Clinical and riological cure of his β lactamase producing GC n after a single oral dose of pivampicillin 2 4 g plus probenecid

olates UL 1–12 were from a female patient (UL) was the regular sexual partner of TB (see above) re his trip to the Far East She had sexual intercourse another partner during TB s stay in the Far East and TB immediately after his return She also admitted genital practice Four days later she developed fever C severe pains in her left wrist and both ankles She had a sore throat Isolates UL 1–2 were isolated r to treatment with pivampicillin 1 4 g three times y for six days She reported clinical cure but four new ates UL 3–6 were obtained during the last day of ampicillin treatment and prior to spectinomycin g im Isolates 7 12 were obtained after this atment Isolates UL 1–4 were from urogenital cimens the rest from the pharynx

lture

The colony morphology typing medium (CMT) scr bed by *Kellogg et al* (15) was used throughout the dy The bacteria were grown for 22 h at 37 °C in 5% O_2 in a hum f ed incubator The β lactamase producing nococci were isolated on Oxo ds Columbia agar pplemented with horse serum and blood and identified described elsewhere (7) All isolates were tested for β-ctamase production with chromatogenic cephalosporin odisk Stockholm Sweden) and for most strains also ith the mod f cation of the *Escam II a* (10) and clover af (24) techniques They were frozen after a minimum subcultures as described previously (8)

ti sera

Ant sera against the MOMP strains were obtained om rabbits and absorbed as described previously (21)

agglut nat on

Reagent staphylococci were prepared as described sewhere (6) During the later phase of the study they ere obtained as a gift from Dr *L Rud n* Pharmacia iagnostics Uppsala Sweden COA tests were perfor ed with heated whole cells (100 °C for 60 m n) as escribed previously (21) and the reactions were graded egative ( ) weak (1 +) moderate (2 +) and strong or ery strong (3 +)

Treatment w th Periodate and Pronase

This was carried out essentially as described by *Angelsen & Maeland* (1) Gonococci were grown for 20–22 h on CMT medium and harvested in 0 01 M phosphate buffered saline (PBS) pH 7 4 spun down at 2 500 × g for 15 min suspended at a concentration of 20 mg ml⁻¹ wet weight boiled in a water bath for 1 h and d vided into four portions One portion was spun down and suspended in four times the original volume with 5 mg ml⁻¹ sod um meta periodate kept in a dark bottle overn ght at + 4 °C spun down and finally resuspended in the original volume of PBS A second portion was treated in the same way as portion one but without periodate A third portion was spun down and resuspended in the original volume of PBS with 0 4 mg ml⁻¹ pronase incubated for 4 h at 37 °C and then heated for 5 min at 100 °C to inactivate the pronase This solution was transparent at the end of the incubation A fourth portion was treated in the same way as portion three but without pronase

Inhibition by Sugars

This procedure was modified from *Maeland* (17) and carried out in two different ways Initially sensitized reagent staphylococci were spun down and resuspended in the same volume of PBS with a 0 28 M sugar solution to be tested and incubated overnight in the cold before the test. A control without sugar was always included It was later found that the same results were obtained by simply mixing one drop of sensitized reagent staphylo-

[illegible]

[illegible] β-lactose lot 66C 0369 D (+) galactose lot 27C 0177 and D (+) glucoseamine lot 25C 0286 (Sigma Chemical Co)

RESULTS

1 Description of COA Regents with Reactivity for the Three Suggested Antigen Classes of the MOMP Reference Strains

In a previous study (21) L RIE results guided the absorptions of anti gonococcal antibodies to be used for coating of reagent staphylococci which in COA tests with the MOMP [illegible]

[illegible] and M The reactivity patterns of the reagents with each of the strains within these classes are shown in Table 2

Class W According to the reactions with prepared reagents strains in this antigen class were divided into three groups group I comprising strains W 16 B 2 G 7 E 5 R 11 D 4 and V 15 group II strains N 10 H 8 C 3 X 9 S 12 T 13 and U 14 and group III strains A 1 and F 6 Strain W 16 reacted with one of the three reagents for W / I and W /II but the antiserum (anti-S 12) used for the W /II group also contained an antibody against a class M antigen Furthermore antiserum against strain W 16 could be absorbed to make it specific for group W /I but not for group W /II Thus W 16 was referred to W/I

Class J Seven of the MOMP strains reacted with the reagents in this class The typical pattern for these strains was that only one or two strains reacted with one of the five class J reagents tentatively designated as indicated in the table

auxotypes and clinical manifestations was demonstrated

We have previously explored the potential use of the co agglutination (COA) method for serological studies of gonococci and the results were strongly correlated to those obtained by indirect immunofluorescence and immunoelectrophoresis (8) Based on the findings by the so called line rocket immunoelectrophoresis (L-RIE) defined reagents could be prepared for COA by selected absorptions When applied to the 16 MOMP reference strains described by *Johnston et al* (14) the results of COA tests with these absorbed antibodies could be referred to at least three classes of antigens We suggested that this might form the basis of a serological classification of gonococci according to a multifactor model

These studies were extended in the present work, and absorbed antibodies were prepared against the MOMP reference strains to identify the three classes of antigens by COA tests These reagents were then used to classify strains used in serological studies by *Maeland* (17) *Geizer* (12) *Apicella* (3) *Arko et al* (4) and *Wang et al* (22) as well as other strains kept at our and other laboratories Included were also β lactamase producing gonococcal strains isolated from six patients

MATERIALS AND METHODS

Neisseria gonorrhoeae *Strains*

One hundred and seventeen gonococcal (GC) s[illegible] were included in the present study They were repres[illegible] ted by six groups of reference strains in all 45 s[illegible] three groups of other defined strains in all [illegible] designations and sources of these strains are shown Table 1 Forty six strains were isolated from six patients infected with β lactamase producing GC

Isolates HCA 1–21 were obtained from a male p[illegible] (HCA) with multiple sexual exposures in the Far E[illegible] They were isolated from urethral and phar[illegible] specimens over a 73-day period due to trea[illegible] failures Isolates HCA 1–2 were from urethra the o[illegible] from the pharynx All isolates except HCA 2 prod[illegible] β lactamase

Isolates BA 1–8 all β lactamase producers w[illegible] obtained from a male patient (BA) with multiple sex[illegible] exposures in the Far East They were isolated over a [illegible] day period BA 1 from the urethra the rest [illegible] pharynx

Isolates RC 1–3 all β lactamase producers were [illegible] a male patient (RC) with a single sexual exposure in the Far East The strains were isolated from ure[illegible] specimens over a 6 day period

Isolate HA 1 was from the urethra of a male p[illegible] (HA) with a history of sexual intercourses in Polan[illegible]

Isolate TB 1 was from a male patient (TB) with [illegible]

TABLE 1 *Compilation of* Neisseria gonorrhoeae *Strains Used in the Present Study*

A Reference Strains *Used by Others in Serological Studies*

1) *Major Outer Membrane Protein (MOMP) Strains* (14) 16 strains obtained from Dr *K H Johnston* Hous[illegible] Texas USA Designations see Table 2
2) *Micro Immuno fluorescence (Micro IF) Reference Strains* (22) eight strains obtained from Drs *K K Ho[illegible]* and *J Knapp* Seattle Washington USA Designations see Table 3
3) *Immunotyping Reference Strains* (4) eight strains obtained from Dr *K H Wong* Atlanta Georgia USA Designations see Table 3
4) *Endotoxin Reference Strains* (17) three strains obtained from Dr *J A Maeland* Trondheim Norw[illegible] Designations see Table 3
5) *Acid Polysaccharide Serogroup Reference Strains* (3) five strains obtained from Dr *M A Apicella* Buffa[illegible] N Y USA Designations see Table 3
6) *Gel Diffusion Reference Strains* (12) five strains obtained from Dr *I Geizer* Prague Czechoslovak[illegible] Designations see Table 3

B Other Defined Strains

1) *Seattle Strains* nine in all six obtained from Drs *K K Holmes* and *J Knapp* three from Dr *T* [illegible] *Buchanan* Seattle Washington USA Designations see Table 4
2) *Other Strains* six in all Four strains described in a previous work (8) one strain 732 from a pat[illegible] [illegible] Southampt[illegible]
3) [illegible] able 4 S[illegible]
3893 isolated from the knee joint and strains He Ge De and Bj from urogenital specimens of five female with DGI Strain K1 was isolated from the pharynx of a female with a history of arthralgia two months af[illegible] intercourse

C Strains from Patient Infected with *β – Lactamase Producing Strains*
Forty six isolates from six patients infected with β – lactamase producing GC were included in the serol[illegible] tests *Histories of these patients* see text

il exposures in the Far East Urethral discharge ed four days after his return to Stockholm and al exposure to patient UL (see below) Clinical and eriological cure of his β-lactamase producing GC n after a single oral dose of pivampicillin 2 4 g plus g probenecid

solates UL 1–12 were from a female patient (UL) was the regular sexual partner of TB (see above) ore his trip to the Far East. She had sexual intercourse another partner during TB s stay in the Far East and h TB immediately after his return She also admitted genital practice Four days later she developed fever °C severe pains in her left wrist and both ankles She had a sore throat Isolates UL 1–2 were isolated or to treatment with pivampicillin 1 4 g three times ly for six days She reported clinical cure but four new lates UL 3–6 were obtained during the last day of ampicillin treatment and prior to spectinomycin g im Isolates 7–12 were obtained after this atment Isolates UL 1–4 were from urogenital ecimens the rest from the pharynx

lture

The colony morphology typing medium (CMT) scribed by *Kellogg et al* (15) was used throughout the udy The bacteria were grown for 22 h at 37 °C in 5 % O_2 in a humified incubator The β lactamase producing nococci were isolated on Oxoids Columbia agar pplemented with horse serum and blood and identified described elsewhere (7) All isolates were tested for β-ctamase production with chromatogenic cephalosporin (Nodisk Stockholm Sweden) and for most strains also ith the modification of the *Escam lita* (10) and clover af (24) techniques They were frozen after a minimum f subcultures as described previously (8)

ntisera

Antisera against the MOMP strains were obtained om rabbits and absorbed as described previously (21)

o agglutination

Reagent staphylococci were prepared as described lsewhere (6) D

lesi ued previously (21) and the reactions were graded iegative (−) weak (1 +) moderate (2 +) and strong or ery strong (3 +)

Treatment with Periodate and Pronase

This was carried out essentially as described by *Angelsen & Maeland* (1) Gonococci were grown for 20–22 h on CMT medium and harvested in 0 01 M phosphate buffered saline (PBS) pH 7 4 spun down at 12 500 × g for 15 min suspended at a concentration of 20 mg ml⁻¹ wet weight, boiled in a water bath for 1 h and divided into four portions One portion was spun down and suspended in four times the original volume with 5 mg ml⁻¹ sodium meta periodate kept in a dark bottle overnight at +4 °C spun down and finally resuspended in the original volume of PBS A second portion was treated in the same way as portion one but without periodate A third portion was spun down and resuspended in the original volume of PBS with 0 4 mg ml⁻¹ pronase incubated for 4 h at 37 °C and then heated for 5 min at 100 °C to inactivate the pronase This solution was transparent at the end of the incubation A fourth portion was treated in the same way as portion three but without pronase

Inhibition by Sugars

This procedure was modified from *Maeland* (17) and carried out in two different ways Initially sensitized reagent staphylococci were spun down and resuspended in the same volume of PBS with a 0 28 M sugar solution to be tested and incubated overnight in the cold before the test A control without sugar was always included It was later found that the same results were obtained by

lot 117C 0046 α lactose lot 14C 1650 β-lactose lot 66C 0369 D (+) galactose lot 27C 0177 and D (+) glucoseamine lot 25C-0286 (Sigma Chemical Co)

RESULTS

1 Description of COA Regents with Reactivity for the Three Suggested Antigen Classes of the MOMP Reference Strains

In a previous study (21) L-RIE results guided the absorptions of anti gonococcal antibodies to be used for coating of reagent staphylococci which in COA tests with the MOMP reference strains of *Johnston et al* (14) gave reactions that fulfilled the criteria for each of the three suggested antigen classes tentatively called W J and M The reactivity patterns of the reagents with each of the strains within these classes are shown in Table 2

Class W According to the reactions with prepared reagents strains in this antigen class were divided into three groups group I comprising strains W 16 B 2 G 7 E 5 R 11 D 4 and V 15 group II strains N 10 H 8 C 3 X 9 S 12 T 13 and U 14 and group III strains A 1 and F 6 Strain W 16 reacted with one of the three reagents for W/I and W/II but the antiserum (anti S 12) used for the W/II group also contained an antibody against a class M antigen Furthermore antiserum against strain W 16 could be absorbed to make it specific for group W/I but not for group W/II Thus W 16 was referred to W/I

Class J Seven of the MOMP strains reacted with the reagents in this class The typical pattern for these strains was that only one or two strains reacted with one of the five class J reagents tentatively designated as indicated in the table

TABLE 2 *Co agglutination Pattern of the MOMP Reference Strains with Reagents for the Three Suggested Antigen Classes Tentatively named W J and M*

Reagent staphylococci coated with absorbed anti GC antibodies	Co agglutination reactions with › MOMP ‹ reference strains																Suggested antigen class pattern
	W 16	B 2	G 7	E 5	R 11	D 4	V 15	N 10	H 8	C 3	Y 9	S 12	T 13	U 14	A 1	F 6	
Anti E 5 abs w N 10	–	3+	3+	3+	3+	1+	2+	–	–	–	–	–	–	–	–	–	
Anti D 4 abs w C 3	2+	1+	1+	1+	3+	3+	3+	–	–	–	–	–	–	–	–	–	W/I
Anti V 15 abs w C 3	–	–	–	–	2+	2+	3+	–	–	–	–	–	–	–	–	–	
Anti N 10 abs w D 4	–	–	–	–	–	–	–	3+	3+	3+	3+	3+	–	–	–	–	
Anti S 12 abs w A 1	2+	–	–	–	–	–	–	3+	3+	3+	3+	3+	3+	3+	–	–	
Anti U 14 abs w R 11	–	–	–	–	–	–	–	2+	2+	2+	2+	–	2+	2+	–	–	W/II
Anti F 6 abs w B 2 & U 14	–	–	–	–	–	–	–	–	–	–	–	–	–	–	2+	3+	W/III
Anti D 4 abs w B 2 & C 3	–	–	–	–	–	3+	3+	–	–	–	–	–	–	–	–	–	J/4
Anti U 14 abs w C 3	–	–	–	–	–	–	–	–	–	–	–	–	3+	3+	–	–	J/14
Anti Y 9 abs w A 1 & B 2	–	–	–	–	–	–	–	–	–	–	2+	–	–	–	–	1+	J/9
Anti S 12 abs w Y 9	–	–	–	–	–	–	–	–	–	–	–	3+	–	–	–	–	J/12
Anti F 6 abs w A 1 & B 2	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	3+	J/6
Anti C 3 abs w A 1	–	2+	3+	2+	3+	3+	3+	2+	3+	3+	–	–	–	–	–	3+	M/a
Anti D 4 abs w B 2	–		–	1+	3+	3+	3+	–	–	3+	–	–	–	–	–	–	M/b
Anti C 3 abs w N 10	–	–	–	–	–	–	3+	–	–	3+	–	–	–	–	–	–	M/c
Anti Y 9 abs w C 3	1+	2+	2+	–	–	–	–	–	3+	–	3+	–	3+	3+	3+	3+	M/d
Anti S 12 abs w H 8	2+	–	–	–	–	–	–	–	–	–	–	3+	–	–	–	–	M/e
Anti E 5 abs w R 11	–	2+	3+	3+	–	–	2+	2+	+	–	–	–	–	–	–	–	M/f
Anti A 1 abs w F 6	–	–	–	–	–	1+	1+	–	–	–	–	–	–	–	2+	–	M/g

antigens responsible for these reactions thus seem to reflect »strain« or »serotype« specificity

Class M The typical patterns for the reagents in this class were reactions with one or more strains in both W/I and W/II, and for some reagents for strains in all three W groups The seven reagents of class M were tentatively designated as M/a g

Based on the suggested antigen class pattern a particular gonococcal strain for example strain J 4 could be classified as W/I J/4, M/a, b Correspondingly, strain X-9 could be classified as W/II J/9 M/d, and strain A-1 as W/III M/d, g

2) *Effect of Treatment with Pronase and Periodate*

Some of the antigens of MOMP strains responsible for Class W reactions were sensitive to pronase and all were resistant to periodate The antigens of the two MOMP strains X-9 and S-12, responsible for J/9 and J/12 reactions, were sensitive to pronase and resistant to periodate The antigens of the other strains in this class were resistant to both pronase and periodate The class M reactive antigens of the MOMP strains were sensitive to periodate and resistant to pronase which was used as an additional criterion for this class

3) *COA Patterns of Selected Reference Strains with Reagents for the Three Antigen Classes*

Twenty nine selected gonoccal strains (for details of source etc see Table 1) were tested by reagent staphylococci for the three tentative antigen classes in Table 2 The antigen class patterns are shown in Table 3

Micro-IF reference strains (Wang et al *(22)*) The reference strains for the Micro-IF groups A, B and C reacted with group W/I W/II and W/III respectively In addition two of the group B reference strains reacted with the W/III reagent and one group C strain with one of W/II reagents All group B and C strains but none of the group A strains reacted with class J reagents All eight reference strains reacted with class M reagents in various combinations

According to these findings the antigen class pattern of strain A_2 could be described as W/I M/a b c strain B_3 as W/II J/12 14 M/e and strain C_1 as W/II III J/6 M/a d

Immunotype reference strains (Arko et al *(4)*) Eight of the n ne refer...

str ...

w ...

reag... which antiserum was also found to contain a class J antibody reacting with this strain Strain 2686 reacted with the W/III and with one of the W/II reagents and strain WJI/1 with another W/II and the W/III reagent. These three strains gave protection only to themselves in guinea-pig chambers, while the other five strains cross reacted in various combinations (4) The close correspondance observed between the class W of COA and the immunotyping system is in agreement with that observed for Micro-IF and the latter system (18)

Four of the strains reacted with class J reagents and all eight with class M reagents in various combinations

Endotoxin reference strains (Maeland *(17)*) The three strains reacted with W/II, III, W/II and W/I, and only one gave a class J reaction

Strain 8551 reacted with factors M/a, b, c, strain V with M/a, d, e and strain VII with M/d All of these reactions were sensitive to periodate and resistant to pronase The M/a reactions for strains 8551 and V ...

galactose, lactose or glucoseamine (Table 5) α- and β lactose had the same blocking effect. The combinations of COA reactions of these three strains with our M/a, b, c, d reagents, their sensitivity to periodate, resistance to pronase and inhibition by sugars correspond to the characteristics of the antigenic factors a_6 a_5 and a_3 described by *Maeland* (16, 17) The M/e reaction was too weak to be reliably tested in the sugar inhibition tests, but other characteristics corresponded to factor a_2 reported by *Maeland*

Acid polysaccharide serogroup reference strains (Apicella *(3)*) The five strains all reacted with W/II and in addition one ...

...reaction of this strain was destroyed by pronase and thus not a true class M reaction Strain 4505/Gc_3 reacted with M/a b, d e, but the reactions M/d and M/e were destroyed by pronase and resistant to periodate, and were thus not true M reactions ...

...city and the results of sugar inhibition tests are shown in Table 5 The sugar inhibition pattern for each M factor corresponds to that found with the endotox...

wi ...

(3 ...

...gel diffusion serotype reference strains (Geizer (12)) Two strains reacted with W/I and three with W/II One strain reacted with a class J reagent and all strains with class M reagents in various

TABLE 2 *Co agglutination Patterns f the MOMP Reference Strains with Reagents for the Three Suggested Antigen Classes Tentatively named W J and M*

Reagent staphylococci coated with absorbed anti GC antibodies	Co agglutination reactions with ›MOMP« reference strains																Suggested antigen class pattern
	W 16	B 2	G 7	E 5	R 11	D 4	V 15	N 10	H 8	C 3	X 9	S 12	T 13	U 14	A 1	F 6	
Anti E 5 abs w N 10	–	3+	3+	3+	3+	1+	2+	–	–	–	–	–	–	–	–	–	
Anti D 4 abs w C 3	2+	1+	1+	1+	3+	3+	3+	–	–	–	–	–	–	–	–	–	W/I
Anti V 15 abs w C 3	–	–	–	–	2+	2+	3+	–	–	–	–	–	–	–	–	–	
Anti N 10 abs w D 4	–	–	–	–	–	–	–	3+	3+	3+	3+	3+	–	–	–	–	
Anti S 12 abs w A 1	2+	–	–	–	–	–	–	3+	3+	3+	3+	3+	3+	3+	–	–	W/II
Anti U 14 abs w R 11	–	–	–	–	–	–	–	2+	2+	2+	2+	–	2+	2+	–	–	
Anti F 6 abs w B 2 & U 14	–	–	–	–	–	–	–	–	–	–	–	–	–	–	2+	3+	W/III
Anti D 4 abs w B 2 & C 3	–	–	–	–	–	3+	3+	–	–	–	–	–	–	–	–	–	J/4
Anti U 14 abs w C 3	–	–	–	–	–	–	–	–	–	–	–	–	3+	3+	–	–	J/14
Anti X 9 abs w A 1 & B 2	–	–	–	–	–	–	–	–	–	–	2+	–	–	–	–	1+	J/9
Anti S 12 abs w X 9	–	–	–	–	–	–	–	–	–	–	–	3+	–	–	–	–	J/12
Anti F 6 abs w A 1 & B 2	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	3+	J/6
nti C 3 abs w A 1		2+	3+	2+	3+	3+	3+	2+	3+	3+	–	–	–	–	–	3+	M/a
nti D 4 abs w B 2	–		–	1+	3+	3+	3+	–	–	3+	–	–	–	–	–	–	M/b
nti C 3 abs w N 10	–	–		–	–	–	3+	–	–	3+	–	–	–	–	–	–	M/c
nti X 9 abs w C 3	1+	2+	2+	–	–	–	–	–	3+	–	3+	–	3+	3+	3+	3+	M/d
nti S 12 abs w H 8	2+	–	–	–	–	–	–	–	–	–	–	3+	–	–	–	–	M/e
nti E 5 abs w R 11	–	2+	3+	3+	–	–	2+	2+	+	–	–	–	–	–	–	–	M/f
nti A 1 abs w F 6		–	–	–	–	1+	1+	–	–	–	–	–	–	–	2+	–	M/g

,nt gen Classes W J and M Reactons Graded as Described in Materials and Methods

		Endotox n ref stra ns[c]			Acid polysaccharide ref strains[d]					Gel diffusion serotype strains[e]				
					1342	1291	4501	8551	P102					
Wx/h	W(I/I)	8551	V	VII	GC_1	GC_2	GC_3	GC_4	GC_5	822/A	554/B	986/C	390/D	509/E
	–	–	–	2+	–			–	–	3+	–	3+	–	–
	–	–	–	–	–	–	–	–	–	–	–	1+	–	–
		–	–	–	–	–	–	–	–	3+	–	3+	–	–
3+	3+		3+	–	3+	3+	3+	–	3+	–	3+	–	3+	3+
3+	–		3+		3+	3+	3+	–	3+	–	3+	–	–	1+
1+	1+	2+	–		3+	2+	2+	2+	2+	–	3+	–	1+	1+
	2+	3+		–	–	–	–	3+	–	–	–	–	–	–
			–	–	–	–	–	–	–	–	–	–	–	–
			–			–		3+	–	–	2+	–	–	–
	–		–		1+	–	1+	–	–	–	–	–	–	–
					2+	1+	2+	–	3+	–	–	–	–	–
		3+				–	–	3+		–	–	–	–	–
	3+	3+	2+		3+	2+	3+	3+	3+	3+	3+	3+	3+	3+
	1+	3+			3+		3+	3+	2+	2+	3+	2+	–	–
		3+						3+	–	–	3+	–	–	–
3+	3+		3+	3+		3+	2+	–		3+	–	3+	3+	–
			?+			1+	3+		2+	1+	1+	–	2+	2+
					3+	–					–	–	–	–
					–				–	–	–	–	3+	–

Ö ebrow stra ns					»Stockholm s ra ns										
EJ	SOA	505	712	P9	14984	15057	15000	11376	14190	23897	He	Ge	Bj	Kl	De
3	3+		3	1	3+			3+	3+	3+	3+	3+	1+	3+	2+
3+	3+		3+		2+	3+		3+	2+	2+	2+	3+			–
3	3+		3+					1+	2+		3+	3+	1+	2+	2+
		1		3+		3+	3								–
		3+		3+		3+	3+								
		3+		3+		3+	3								–
					1							–			
	1			2+		3+									
		1		1+											
				3+		3+									
															–
	3+	3+		2+	3+	3+	3+	3+		3+	3+	3+	3+	3+	
	3	1		1	1+	3+	3+	3+		3+	3+	3+		3+	
		1+		1+				3+		3+	?+	?+	2+	3+	
	3	3+	3	3+		1+	3+		3+			3+	3+	3+	
	2				2+	1+									1+
						1+	3+	3+					3+	–	
				1+								–			

TABLE 3 *Co agglutination Patterns of 29 Selected Reference Strains with Reagents for the Three Tenta*

Reagent staphylococci coated with absorbed anti GC antibodies	Tentative antigen class pattern	Micro IF reference strains[a]								Immunotype referen			
		4286 (A_1)	1859 (A_2)	1567 (A_3)	5293 (B_1)	5288 (B_2)	5001 (B_3)	5016 (C_1)	1955 (C_2)	A/a	C/c	G/d	N
Anti E 5 abs w N 10		3+	3+	3+	–	–	–	–	–	–	–	3+	
Anti D 4 abs w C3	W/I	3+	3+	3+	–	–	–	–	–	–	–	3+	
Anti V 15 abs w C 3		3+	3+	3+	–	–	–	–	–	–	–	3+	
Anti N 10 abs w D 4		–	–	–	–	3+	2+	–	–	3+	3+	–	3
Anti S 12 abs w A 1	W/II	–	–	–	–	3+	3+	3+	–	3+	–	3+	3
Anti U 14 abs w R 11		–	–	–	3+	3+	2+	–	–	2+	–	–	3
Anti F 6 abs B 2&U 14	W/III	–	–	–	3+	3+	–	3+	3+	–	1+	–	
Anti D 4 abs w B 2&C 3	J/4	–	–	–	–	–	–	–	–	–	–	–	
Anti U 14 abs w C 3	J/14	–	–	–	1+	1+	2+	–	–	–	–	–	
Anti X 9 abs w A 1&B 2	J/9	–	–	–	–	–	–	–	–	–	–	–	
Anti S 12 abs w X 9	J/12	–	–	–	–	–	2+	–	–	2+	–	3+	
Anti F 6 abs w A 1&B 2	J/6	–	–	–	–	2+	–	3+	3+	–	–	–	
Anti C 3 abs w A 1	M/a	3+	3+	3+	–	3+	1+	2+	3+	3+	3+	3+	3
Anti D 4 abs w B 2	M/b	–	3+	3+	1+	3+	–	–	–	–	–	1+	
Anti C 3 abs w N 10	M/c	–	2+	2+	–	3+	–	–	–	–	–	–	1
Anti X 9 abs w C 3	M/d	–	–	–	3+	3+	1+	3+	–	3+	–	3+	
Anti S 12 abs H 8	M/e	1+	–	–	2+	1+	3+	–	–	2+	–	3+	
Anti E 5 abs w R 11	M/f	3+	–	3+	–	–	–	–	3+	1+	–	–	3
Anti A 1 abs w F 6	M/g	–	–	–	3+	1+	–	1+	–	–	–	–	

a) *Wang et al* see ref (22) b) *Arko et al* see ref (4) c) *Maeland* see ref (17) d) *Apicella* see ref (3) e) *Gei er* see

TABLE 4 *Co agglutination Patterns of 26 Defined Gonococcal Strains Obtained from Seattle Örebro and Stockh*

Reagent staphylococci coated with absorbed *anti GC antibodies*	*Tentative antigen class pattern*	*»Seattle« strains*							
		5029	*5010*	*5030*	*5064*	*6218*	*F62*	*Black*	C 33
Anti E 5 abs w N 10		3+	–	3+	3+		–	–	–
Anti *D 4* abs w *C 3*	W/I	3+	3+	3+		–	–	–	–
Anti V 15 abs w C 3		2+		3+	–	–	–	–	–
Anti N 10 abs w D 4		–	–	–	–		–	3+	–
Anti S 12 abs w A 1	W/II	–	–	–	–	3+		3+	3+
Anti U 14 abs w R 11		–	–	–	–	3+	2+	2+	3+
Anti F 6 abs w B 2 & U 14	W/III	–	–	–	–	–	3+	–	–
Anti D 4 abs w B 2 & C 3	J/4	–	3+	–	–			–	–
Anti U 14 abs w C 3	J/14	–	–		–	3+	1+	3+	3+
Anti X 9 abs w A 1 & B 2	J/9	–	–		–	–	–	–	–
Anti S 12 abs w X 9	*J/12*	–	–		–	–	–	2+	–
Anti F 6 abs w A 1 & B 2	J/6	–	–	–	–	–	–		–
Anti C 3 abs w A *1*	M/a	3+	3+	3+	3+	–	–	3+	–
Anti D 4 abs w B 2	M/b	2+	3+	3+	2+	–	1+	3+	–
Anti C 3 abs w N 10	M/c	2+	–	–	1+	–	–	–	–
Anti X 9 abs w C 3	M/d	3+	–	–	2+	3+	3+	1+	2+
Anti S 12 abs w H 8	M/e	2+	–	3+	–	*1+*	*1+*	3+	–
Anti E 5 abs w R 11	M/f	–	3+	–	3+	–	–	3+	–
Anti A 1 abs w F 6	M/g	2+	1+	1+	–	–	1+	–	–

) *COA Patterns of Defined Strains from Various ources*

The reactivity patterns for these 26 strains are hown in Table 4 These strains were from aboratories in Seattle Örebro and Stockholm

Seattle stra ns Five reacted with W/I three vith W/II and one with W/II III Four reacted vith one or two class J reagents and all reacted with lass M antisera in various combinations

Strains NRL 5029 5010 5030 5064 and 6218 were non typeable in the Micro-IF (22) The first our reacted with W/I NRL 5029 was used to ibsorb all mouse antisera, which could explain the non reactivity of the other three in Micro-IF NRL 5218 reacted in group W/II

Örebro stra ns Four strains reacted with group W/I and two with group W/II Only strain P9 reacted strongly with a class J reagent Each of the trains 670 EJ SOA and 505 were shown previously to have strain specific antigens (8) A weak class J reaction was observed for SOA while 670 EJ 505 and 732 did not react in this class Strain 732 has been found to be antigenically similar to strain EJ in COA and IE studies with anti EJ and anti 732 respectively This is confir med by results presented in Table 4

Stockholm stra ns Nine strains reacted with W/I reagents Two strains reacted with W/II and one of these with one W/I reagent. However the antiserum used to prepare the W/I reagent was also found to contain a class J antibody against this strain

Strains 14894 15057 15000 11376 and 14190 were shown previously to have »strain« specific antigens but only one reacted with the class J reagents available

5) COA Patterns with Strains Presumed to be Identi cal but Obtained from Different Sources

The two strains 8551 were obtained indepen dently from Dr *Maeland* and Dr *Apicella* (Table 4) Both gave the same reactions with six of the reagents for classes W J and M The strain from Dr *Apicella* gave one additional class J reaction

Strain NRL 6218 (C 33) (22) and C 33 (5) were obtained independently from Drs *Holmes and Knapp* and from Dr *Buchanan* and they gave four reactions with the same class W J and M reagents

Strain F62 (5) was obtained from Dr *Buchanan* (Table 4) from Drs *Holmes and Knapp* as NRL 5293 (Table 3) and from Dr *Wong* as KF/f (4) (Ta ble 3) The two first gave very similar reactions while KF/f clearly differed from the other two

Strain 2686/g (4) (Table 3) was obtained from

rformed w h reagents for Three Ant gen Classes W J and M React ons Graded as Descr bed n Materials and Meth ods

	Pa en BA Iso a e No					Pat ent RC Iso a e No			Pa ent HA (one solate)	Pa en TB (one solate)	Pa ent LL Isolate No		
	3	4	5 6	7	8	1	2	3			1 2	3 6	7 12
										3 +	3 +	3 +	3
										1 +	3 +		/ +
										3 +	1 + / 3 +	3 +	3 +
									1				
3	3	3	3	3	3	3	3	3 +					
									2				
									3 +				
2		2							3				
3	3	1	1	1	3	3	3	3					
									3 +				
	2		2	3 +	3 +	3	3 +	3	3 +				
				3	3 +	3 +	3 +	3 +	2 +	3 +	3 +	3	3
				1 +	1 +	3 +	3 +	3 +		1	2 +	1 +	1
3	2	3	2										
1	1	1							3 +	3 +			
						2			2 +		3 +	3 +	3 +
											1		
1	1	1				1 +	1 +	1 +					
													–

TABLE 5 *Co-agglutination Patterns of the »Endotoxin« and »Acid Polysaccharide« Reference Strains with Class Reagents Effect of Treatment with Pronase and Periodate Blocking of the Reactions with Sugars*

Reagent staphylococci coated with absorbed anti-GC antibodies	Tentative antigen class pattern	Endotoxin reference strains			Acid polysaccharide reference [strains]			
		8551	V	VII	1342 GC_1	1291 GC_2	4505 GC_3	8551 GC_4
Anti C-3 abs w A-1	M/a	r[a]/s[b](L)[c]	r/s (L)	–	r/s (L)	s/s	r/s (GL)	r/s (L) r
Anti D 4 abs w B-2	M/b	r/s (L)	–	–	r/s (GL)	–	r/s (GL)	r/s (L)
Anti C-3 abs w N-10	M/c	r/s (GL)	–	–	–	–	–	r/s (GL)
Anti X-9 abs w C-3	M/d	–	r/s (NS)	r/s (NS)	–	r/s (NS)	s/r	–
Anti S-12 abs w H-8	M/e	–	r/s (nd)	–	–	r/s (nd)	s/r	–
Anti E-5 abs w R-11	M/f	–	–	–	r/s (NS)	–	–	–
Anti A-1 abs w F-6	M/g	–	–	–	–	–	–	–

a) Effect of treatment with pronase r = resistant s = sensitive
b) Effect of treatment with periodate r = resistant, s = sensitive
c) Blocking of reactions with sugars
L = blocking of the reaction with lactose
GL = blocking of the reaction with galactose and lactose
NS = no blocking of the reaction with glucose, galactose, lactose or glucoseamine
nd = not done

combinations Strains 882/A and 986/C were very similar, as were 390/D and 509/E All strains were used by *Geizer* to absorb all heterologous antisera Unique reactions are found only for 554/B in class J and M for strain 390/D in class M

TABLE 6 *Co-agglutination Patterns of β-Lactamase Producing Gonococcal Strains Isolated from Six Patients Th*

Reagent staphylococci coated with absorbed anti GC antibodies	Tentative antigen class pattern	Patient HCA Isolate No						
		1	2	3 14	15	16	17	18
Anti E 5 abs w N 10 Anti D 4 abs w C 3 Anti V 15 abs w C 3	W/I	–	–	–	–	–	–	–
Anti N 10 abs w D 4 Anti S 12 abs w A 1 Anti U 14 abs w R 11	W/II	3 +	3 +	3 +	3 +	3 +	3 +	3 +
Anti F 6 abs w B 2&U 14	W/III	–	–			–	–	–
Anti D 4 abs w B 2&C 3	J/4	–	–	–		–	–	–
Anti U 14 abs w C 3	J/14	–			–	–	–	–
Anti X 9 abs w A 1&B 2	J/9			–	–		–	–
Anti S 12 abs w X 9	J/12	2 +	2 +	3 +	3 +	3 +	3 +	3 +
Anti F 6 abs w A-1&B 2	J/6	–	–	–	–	–		–
Anti C 3 abs w A 1	M/a	1 +	3 +	1 +	3 +	–	–	2 +
Anti D 4 abs w B 2	M/b	3 +	–	1 +	–	–	–	–
Anti C-3 abs w N 10	M/c	7 +	–	2 +	–	–	–	–
Anti X 9 abs w C 3	M/d	–	–	–	3 +	3 +	3 +	3 +
Anti S 12 abs w H 8	M/e	–	–	–a)	–	–	–	–
Anti E 5 abs w R 11	M/f	–	3 +	–b)	–	–	2 +	3 +
Anti A-1 abs w F 6	M/g	–	–	–	–	–	–	–

a) Isolate No 5 gave a 2 + reaction
b) Isolate No 13 gave a 2 + reaction

16 D 4 and V 15 which we placed in W/I Our

lassify strains ...

... be due to the fact that strain NRL-5029 was

Micro IF by *Mark & Wang* (18) Furthermore COA tests discriminated between the strains that gave protection only for the homologous strain in the guinea pig chamber model and between the ... protection (4) It was also of

Our findings in this and a previous work (21) ...icated that there was a sharp antigenic demarca...n between the W/I and W/II groups However ...thin the groups there seemed to be a gradual ...der of relatedness as judged on the basis of ...glutinating properties and groups of closely ...ated strains absorbed out the antisera of each ...her We therefore suggest that the group W/I and ...oup W/II reactions are due to two different alter...tive structures Pronase digestion experiments in...cated that they are basically of protein nature al...ough highly protected against proteolytic enzy...es which is analagous to the meningococcus ...ajor outer membrane proteins (11) We suggest ...at each structure may vary to a limited extent and ...rry multiple antigenic sites The variations within .../I would then be represented by strains W 16 ...2 G 7 E 5 R 11 D-4 V 15 Correspondingly ...e variations within W/II would be represented by ...10 H 8 C 3 X 9 S 12 T 13 U 14 The strains ...ithin W/III might be included in W/II However ...rational subgrouping of the class W groups must ...wait biochemical characterization of these anti...gens

Some of the class J reactions might well fit into class W for example the reactions of D 4 and V 15 T 13 and U 14 and F-6 which are pronase stable Many of the class J reactions have also been found to be dependent on colony morphology (9) The possible relationship of class J to the MOMP serotype system of *Johnston et al* (14) is therefore not clear at present

The class M reactions were found to be correlated with both the *endotoxin* (17) and the acid polysaccharide systems (3) The formulas presented in this ...rains should however be taken with caution since some of the reagents had an additional activity against pronase sensitive antigens It was of interest to note that strain 1342 (GC$_1$) used for absorptions by *Apicella* (3), contained more than one M factor *Sadoff et al* (20) showed by solid phase radioimmunoassay that LPS and the acid polysaccharide of GC$_1$ had a broad reactivity Noteworthy was also the broad reactivity within class M of strain NRL-5029 which was considered to be an antigenically unique gonococcus strain by *Wang et al* (22) Repeated isolates of β lactamase producing gonococci showed stable reactions for class W and class J while there were considerable variations in the class M patterns

The nomenclature suggested in this paper should be considered to be tentative There will certainly be a need to harmonize the Micro-IF groups and the COA class W groups when more information is obtained about these antigens We have avoided capital letters since these are used for the capsular antigens of *N meningitidis* For class J we have adopted an operational classification with arabic numerals depending on the reaction pattern seen with the MOMP reference strains However as suggested above this class may turn out to be very heterologous Arabic numerals for protein antigens analagous to those of *N meningitidis* would still be useful Finally we have used small letters for the class M factors

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Dr *Wong* and as A-1 (14) from Dr *Johnston* (Table 1) The COA reactions obtained with A-1 were also found for 2686/g which had one additional reaction in classes W, J and M It will be seen that the complete reaction pattern of this strain was very similar to that of the two F62 strains from Seattle

The close relationship between NRL 5293 (F62) and 2686/g and their difference from the KF/f (F62) was also observed by Micro IF (18)

6) *COA Patterns with Strains from Patients with Disseminated Gonococcal Infection*

Strain WJ 1/1 (Table 3) is reported to be isolated from a heart valve (4) Strains 670, 732, 23892, De He, Ge and Bj were all isolated from patients with DGI Strain NRL 7122 is used for the isolation of a principal outer membrane protein found in most strains causing DGI (13) All these strains are reactive with W/I except WJ 1/1 which reacts with W/II This is of interest, since DGI strains are referred to groups A_2 and A_3 in the Micro-IF (22), and strain WJ 1/1 is reported to react with group B (18)

7) *COA Patterns with β-Lactamase Producing Strains*

From six patients, without possibility of reinfection, β-lactamase producing gonococci were isolated during the period of the study The serological patterns of 46 isolates from these patients are shown in Table 6

Three patients (HCA, BA and RC) infected January-March 1978 in the Far East, had the same W/II pattern which was different from that of a patient (HA) infected in Poland, and a patient (TB) infected in the Far East one year later

Twenty-one isolates were obtained from patient HCA over a 73 day period due to repeated treatment failures of his pharyngeal gonorrhoea All these isolates had the same class W and J reactions However there were obvious variations in the class M patterns The first urethral isolate prior to pivampicillin produced β lactamase while the urethral isolate after treatment did not These two isolates had different class M patterns The β-lactamase producing pharyngeal isolate HCA 3 had the same class M pattern as the initial urethral isolate and this was also true for the subsequent 11 pharyngeal isolates With isolate HCA 15, after 53 days a continous change was noted in the class M reactions which could not be correlated with treatment attempts

The different class J and M reactions in the pharyngeal isolates BA 2 and 4 represented strains with an antibiogram markedly different from the urethral isolate BA 1 and the pharyngeal isolates BA 3, 5 and 6 However, isolates BA 2 and 3 w obtained at the same time from different pharyn swabs as BA 4 and 5 Isolates BA 7 and 8 at different times had antibiograms of the BA 1 BA 2 types, respectively, but differed in the way from all previous isolates in the class reactions Thus, in these two patients with suspicion of an infection with more than one the class M reaction patterns showed variations repeated isolates

The Swedish consort UL of TB was found have β-lactamase producing gonococci with same class W and M pattern as TB The reactivity of UL 3–6 with the second W/I was confirmed with absorbed antibodies three other strains This change occured treatment with pivampicillin which cured symtoms The initial COA pattern recurred in 7–12 after treatment with spectinomycin

DISCUSSION

In the present work we have extended studies of the serologic classification of *Neiss gonorrhoeae* by co agglutination (8, 21) with closer examination of the three classes of antig that we proposed previously (21) and tentati named W J and M Antigen class W was foun correspond well with the three groups in the M IF system described by *Wang et al* (22), and the immunotyping system of *Arko et al* Antigen class J conforms to some extent with MOMP serotype system of *Johnston et al* (1 while antigen class M is closely related to endotoxin and acid polysaccharide systems *Maeland* (17) and *Apicella* (3) We have found agglutination to be a rapid easy and reproduc method for classification of gonococci along th lines Moreover it can be used to study sensitivity of antigens to proteolytic enzymes a oxidizing agents and to study the inhibition sugars of the reactivity of polysaccharide antige

Most of the MOMP reference strains were tes in the Micro IF system by *Wang* (personal comm nication) He found that strains B 2 G 7 E 5 a R 11 belonged to group A while strains W-16 D and V-15 could not be classified Strains N-10 H C-3, X-9 and A-1 belonged to group B while str F 6 was placed in group C Strain T-13 was fou to be intermediate between groups B and C but w finally placed in group B Most of the MOM reference strains were thus classified analogously Micro IF and COA with the exception of stra A-1 which we referred to W/III and strain

√ 16 D-4 and V 15 which we placed in W/I Our ailure to place strain A 1 in group W/II instead of group W/III could be due to the cross reactivity of the Micro-IF group B and C reference strains seen with the available COA reagents Wang s failure to classify strains W 16 D 4 and V 15 by Micro-IF could be due to the fact that strain NRL-5029 was used as absorbing strain by *Wang et al* (22) and this strain reacted with our W/I reagents

COA of the immunotyping strains of *Arko et al* (14) gave a pattern similar to that obtained with Micro IF by *Mark & Wang* (18) Furthermore COA tests discriminated between the strains that gave protection only for the homologous strain in guinea pig chamber model and between the ains that gave cross protection (4) It was also of

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ough highly protected against proteolytic enzy es which is analagous to the meningococcus ajor outer membrane proteins (11) We suggest at each structure may vary to a limited extent and rry multiple antigenic sites The variations within /I would then be represented by strains W 16 2 G 7 E 5 R 11 D 4 V 15 Correspondingly e variations within W/II would be represented by 10 H 8 C 3 X 9 S 12 T 13 U 14 The strains ithin W/III might be included in W/II However rational subgrouping of the class W groups must wait biochemical characterization of these anti ens

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SEROLOGY OF *NEISSERIA GONORRHOEAE* DEMONSTRATION BY CO-AGGLUTINATION AND IMMUNOELECTROPHORESIS OF ANTIGENIC DIFFERENCES ASSOCIATED WITH COLOUR/OPACITY COLONIAL VARIANTS

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Danielsson D & Sandstrom E Serology of *Neisseria gonorrhoeae* Demonstration by co-agglutination and immunoelectrophoresis of antigenic differences associated with colour/opacity colonial variants Acta path microbiol scand Sect. B 88 39–46 1980

Serological classification of *Neisseria gonorrhoeae* by co agglutination (COA) into previously described antigen classes W and J was confirmed in the present work Immunization of rabbits with classified organisms gave antibodies which produced the expected results when used in the preparation of COA reagents Colour/opacity colonial variants of isogenic strains characterized by stereomicroscopy as opaque and transparent respectively were found to influence immunization absorption co-agglutination and precipitation in immunoelectrophoresis (IE) It was shown by COA and IE that organisms of opaque colonies often contained an extra antigenic factor(s) which resisted heating at 100 °C but was sensitive to treatment with pronase They were extracted by heating the organisms in saline or lithium chloride solution Serological classification with COA of clinical isolates was reproducible with reagents for antigen classes W and J Colony morphology dependent reactions mostly due to organisms of opaque colonies occurred in 7% with reagents for antigen class W and in 20% for antigen class J

Key words *Neisseria gonorrhoeae* co-agglutination immunoelectrophoresis serology

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SEROLOGY OF *NEISSERIA GONORRHOEAE* DEMONSTRATION BY CO-AGGLUTINATION AND IMMUNOELECTROPHORESIS OF ANTIGENIC DIFFERENCES ASSOCIATED WITH COLOUR/OPACITY COLONIAL VARIANTS

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Danielsson D & Sandstrom E Serology of *Neisseria gonorrhoeae* Demonstration by co-agglutination and immunoelectrophoresis of antigenic differences associated with colour/opacity colonial variants Acta path microbiol scand Sect B 88 39–46 1980

Serological classification of *Neisseria gonorrhoeae* by co agglutination (COA) into previously described antigen classes W and J was confirmed in the present work Immunization of rabbits with classified organisms gave antibodies which produced the expected results when used in the preparation of COA reagents Colour/opacity colonial variants of isogenic strains characterized by stereomicroscopy as opaque and transparent, respectively were found to influence immunization absorption co-agglutination and precipitation in immunoelectrophoresis (IE) It was shown by COA and IE that organisms of opaque colonies often contained an extra antigenic factor(s) which resisted heating at 100 °C but was sensitive to treatment with pronase They were extracted by heating the organisms in saline or lithium chloride solution Serological classification with COA of clinical isolates was reproducible with reagents for antigen classes W and J Colony morphology dependent reactions mostly due to organisms of opaque colonies occurred in 7% with reagents for antigen class W and in 20% for antigen class J

Key words *Neisseria gonorrhoeae* co-agglutination immunoelectrophoresis serology

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By means of indirect immunofluorescence co-agglutination (COA) and immunoelectrophoresis we have previously demonstrated »strain-specific« interstrain cross reactive and common antigens of whole or disintegrated organisms of various *Neisseria gonorrhoeae* strains (9 10) including the so-called major outer membrane protein (MOMP) reference strains of *Johnston et al* (6) COA reagents were prepared against these strains with selectively absorbed antibodies which permitted separation of the MOMP three different antigen classes tentatively named W J and M (10) A wide range of gonococcal strains used in serological studies by other authors were classified by these COA reagents (10) Strains isolated from patients with disseminated gonococcal infection (DGI) reacted consistently with reagents for one and the same of the three groups of antigen class W This classification was found to be reproducible in tests with identical strains obtained from various laboratories and also with β lactamase producing strains isolated on several occasions from the same patient

TABLE 1 *COA Reactions Obtained with MOMP and Micro-IF Reference Strains and with C*

Reagent staphylococci coated with absorbed anti GC antibodies	Tentative antigen class pattern	MOMP reference strains W-16	B 2	G-7	E-5	R-11	D 4	V-15	N 10	H 8	C 3	[illegible]
Anti F-5 abs w N-10		–	3+	3+	3+	3+	1+	2+	–	–	–	
Anti D 4 abs w C-3	W/I	2+	1+	1+	1+	3+	3+	3+	–	–	–	
Anti V-15 abs w C-3		–	–	–	–	2+	2+	3+	–	–	–	
Anti N-10 abs w D 4		–	–	–	–	–	–	–	3+	3+	3+	3
Anti S-12 abs w A-1	W/II	–	–	–	–	–	–	–	3+	3+	3+	3
Anti U-14 abs w R-11		–	–	–	–	–	–	–	2+	2+	2+	2
Anti F 6 abs w B-2 & U-14	W/III	–	–	–	–	–	–	–	–	–	–	
Anti 670 Op abs w P9 Op		–	2+	2+	2+	3+	3+	1+	–	–	–	
Anti 670 Op abs w P9 Tr		–	2+	2+	2+	3+	3+	1+	–	–	–	
Anti 670 Tr abs w P9 Op	W/I	1+	1+	1+	2+	2+	1+	–	–	–	–	
Anti 670 Tr abs w P9 Tr		–	2+	1+	1+	2+	2+	–	–	–	–	
Anti 670 Op abs w SOA Op/Tr[c]	J/12	–	–	–	–	–	–	–	–	–	–	
Anti 670 Op abs w 670 Tr		–	–	–	–	–	–	–	–	–	–	
Anti SOA Op abs w P9 Op/Tr		3+	3+	3+	3+	3+	3+	3+	–	–	–	
Anti SOA Tr abs w P9 Op	W/I	3+	3+	3+	3+	3+	2+	1+	–	–	–	
Anti SOA Tr abs w P9 Tr		1+	3+	3+	3+	3+	2+	–	–	–	–	
Anti P9 Op abs w 670 Tr		–	2+	–	–	2+	–	–	2+	2+	1+	1
Anti P9 Tr abs w SOA Op	W/II	–	–	–	–	–	–	–	2+	2+	2+	1
Anti P9 Tr abs w SOA Tr		–	–	–	–	–	–	–	2+	3+	3+	3

a) Op = Opaque b) Tr = Transparent c) = reaction resistant to pronase d) = reaction sensitive to pronase

In a previous work minor variations in the COA reactions were noted with some strains (3) In the present study it is shown that such variations can be ascribed to antigenic differences associated with colour/opacity variants of isogenic strains The reproducibility of the COA method for serological classification of *Neisseria gonorrhoeae* is investigated as well as the incidence of reactions dependent on colony morphology variants

MATERIAL AND METHODS

Neisseria gonorrhoeae *Strains and Culture*

Eighty five gonococcal (GC) strains were included in the present study 27 of which were represented by the 16 major outer membrane protein (MOMP) serotype strains (designations see Table 1) *described by Johnston* [illegible] reference strains of [illegible] (14) and the [illegible] h were used by us in our previous serological studies of GC (3 10) Fifty-eight strains were unselected clinical isolates from male and female patients attending the out patient departments for veneral diseases at Central C[illegible] Hospital Örebro and at Sodersjukhuset Stock[illegible] Sweden (34 of the strains were from two or urogenital and/or rectal sites in 14 patients) A[illegible] strains were isolated and identified as described [illegible] viously (1 2)

The GC organisms for use in COA tests were cul[illegible] for 18–22 h at 35–36 °C in candle jars or in a CO[illegible] thermostat on the colony morphology typing me[illegible] (CMT) described by *Kellogg et al* (7) The c[illegible] morphology of the MOMP strains has been desc[illegible] previously (9) The Micro IF strains consisted of o[illegible] T2 colony variants except A_3 and B_1 which had [illegible] accepted as colony variants resembling T3 Opaque [illegible] transparent variants of colony morphology type T2 [illegible] selected according to criteria described by *Swanson* [illegible] These T2 colonies showed distinct edges by st[illegible] *microscopy Those which were dark brown or very* [illegible] in colour by diffusing substage mirror were *opaque* [illegible] by polished mirror and those which were light or [illegible] brown by *diffusing mirror were transparent* [illegible] polished mirror Both opaque and transparent [illegible] colonies showed double highlights as described by [illegible] *et al* (8) when illuminated from above at an angle o[illegible]

...iriants of Three Isogenic Strains Reactions Graded as Described in Material and Methods

			Col /opac. colonial variants						Micro IF reference strains							
			670 T2		SOA T2		P9 T2		4286	1859	1567	5293	5288	5001	5016	1955
U 14	A 1	F 6	Op[a)]	Tr[b)]	Op	Tr	Op	Tr	(A_1)	(A_2)	(A_3)	(B_1)	(B_2)	(B_3)	(C_1)	(C_2)
–	–	–	3 +	3 +	3 +	3 +	–	–	3 +	3 +	3 +	–	–	–	–	–
–	–	–	3 +	3 +	3 +	3 +	–	–	3 +	3 +	3 +	–	–	–	–	–
–	–	–	2 +	3 +	3 +	3 +	–	–	3 +	3 +	3 +	–	–	–	–	–
–	–	–	–	–	–	–	3 +	3 +	–	–	–	–	3 +	2 +	–	–
3 +	–	–	–	–	–	–	3 +	3 +	–	–	–	–	3 +	3 +	3 +	–
2 +	–	–	–		–	–	3 +	3 +	–	–	–	3 +	3 +	3 +	–	–
–	3 +	3 +	–	–	–	–	–	–	–	–	–	3 +	3 +	–	3 +	3 +
	–	–	3 + c)	1 +	3 +	2 +	–	–	2 +	2 +	2 +	–	–	–	–	–
–	–	–	3 +	2 +	3 +	3 +	–	–	3 +	3 +	3 +	–	–	–	–	–
–	–	–	3 +	1 +	3 +	2 +	–	–	3 +	2 +	1 +	–	–	–	–	–
–	–	–	2 +	–	3 +	1 +	–	–	3 +	2 +	2 +	–	–	–	–	–
–	–	–	3 + d)	–	–	–	–	–	–	–	–	–	–	–	–	–
–		–	3 + d)	–	–	–	–	–	–	–	–	–	–	–	–	–
–		–	3 +	3 +	3 +	3 +	–	–	3 +	3 +	3 +	–	–	–	–	–
	–	–	3 +	3 +	3 +	3 +	–	–	3 +	3 +	3 +	–	–	–	–	–
–	–	–	3 +	3 +	3 +	3 +			3 +	3 +	3 +	–	–	–	–	–
–	–	–	–	–	3 + d)	–	3 +	2 +	3 +	–	–	–	1 +	2 +	–	2 +
		–	–	–	–	–	3 +	3 +	–	–	–	–	2 +	2 +	–	2 +
2 +	–	–	–	–	–	–	3 +	3 +	–	–	–	–	3 +	3 +	–	3 +

/Tr indicates separate absorptions with organisms of opaque and transparent colonies respectively

45 °C With this illumination the opaque colonies ... a whitish colour and the transparent ones were ...ish greyish on ocular inspection when the CMT plate ...s placed on the glass plate of the stereomicroscope and ...uminated from below Strains were preserved as ...scribed previously (3)

...tisera

Antisera against the MOMP strains were obtained ...om *rabbits as described previously (9) Antisera* against ...C cells of *T2 opaque* and transparent colonies of the ...C *strains 670 SOA* and P9 were obtained by ...mmunizing rabbits with formalin fixed *whole cells (3)* ...wo for each of the opaque and transparent colony ...orphology variants *The first dose* ...
...eou
...l–5 ...
...The ...
A
...ally

Add... absorptions were carried out with *heated whole cells* (see below) for 3–4 h at 37 °C for each of the antisera against opaque or transparent colony variants of GC 670 SOA *and P9*

Co agglutination

The production stabilization and coating of protein *A-containing* staphylococci with antibodies and the *performance of the COA* tests followed the procedures described previously (1 3) Whole *GC cells heated* at *100 °C for 30* or *60* min and in some experiments lithium chloride or saline extracts (see below) were used as test antigens Negative reactions were graded – weak 1 + *moderately* strong *2 +* and strong or very strong reactions *3 +*

... were performed as described previously *(3) The* supernatant of sonicated organisms (200 mg ml⁻¹ wet weight) (3) saline or lithium chloride extracts (see *below*) were used as antigens

Preparation of Antigen Extracts

GC organisms were suspended in *sterile 0 15 M* saline at a concentration of 200 mg ml⁻¹ and boiled in water bath for 30 min as described *for preparation* of meningococcal group *B* serotype antigens (4) The

supernatant was collected after centrifugation at 3 000 × g for 20 min Correspondingly the GC organisms were suspended in 0 2 M LiCl –0 1 M Na acetate pH 5 6 and treated for 2 h at 50 °C during vigorous stirring on a magnetic stirrer (5) The suspension was centrifuged at 3 000 × g for 20 min and the supernatant collected Both this lithium chloride extract and the saline extract were used in some of the COA and C LIE & R LIE tests as described under Results

Treatment with Pronase and Periodate

Whole cells heated at 100 °C were treated with pronase and periodate as described previously (10)

RESULTS

1) *Co agglutination Reactions with Colour/Opacity Colonial Variants*

Organisms of the MOMP and Micro IF reference strains and of colour/opacity variants of three isogenic GC strains were tested with COA reagents for antigen class W (10) and with reagent staphylococci coated with absorbed antibodies for the variants of the three isogenic strains Pertinent COA results are shown in Table 1

Strains 670 and SOA reacted with the reagents for antigen group W/I and strain P9 with W/II The results were similar for the colour/opacity colonial variants of the isogenic strains It can also be seen that strains 670 and SOA reacted with reagents corresponding to group A strains and P9 with reagents for group B strains of the Micro IF system of *Wang et al* (14) Correspondingly reagent staphylococci coated with anti 670 or a[illegible] SOA absorbed with P9 reacted with the MO[illegible] strains of antigen group W/I and group A stra[illegible] the Micro IF system and those of anti P9 ab[illegible] with 670 or SOA with the MOMP stra[illegible] antigen group W/II and with two of the gr[illegible] and one of the group C strains

Table 1 shows however that these rea[illegible] patterns were dependent on the colour/o[illegible] colonial variants of the organisms used for im[illegible] zation absorption and co agglutination Th[illegible] anti 670 Op absorbed with P9 Op or Tr g[illegible] reactions with the MOMP strains of antigen g[illegible] W/I and with the group A strains of Micro IF expected but in addition also with strain S 1[illegible] group W/II Anti 670 Tr absorbed with P9 O[illegible] Tr reacted with group W/I strains but no rea[illegible] was detected with S 12 This strain also re[illegible] with anti 670 Op absorbed with SOA Op or Tr a[illegible] even after absorption with 670 Tr It can be s[illegible] that these reagents also reacted with organism[illegible] 670 Op colonies but not with those of Tr or[illegible] any of the MOMP or Micro IF strains T[illegible] reaction was found to be sensitive to pronase in t[illegible] same way as a corresponding colony morphol[illegible] dependent reaction noted for SOA Op with anti [illegible] Op absorbed with 670 Tr This reagent reacted w[illegible] MOMP strains of group W/II but also with stra[illegible] B 2 and R 11 of W/I and with one group C a[illegible] two group B strains of Micro IF in addition t[illegible] strain 4286 of group A Anti P9 Tr did not rea[illegible] with B 2 R 11 or 4286 regardless of absorb[illegible] strain but reacted with group W/II and group [illegible] and C strains

TABLE 2 *Demonstration by Crossed line or Rocket line Immunoelectrophoresis of Antigenic Differ[illegible] Preparations from Colour/Opacity Colonial Variants of Isogenic Strains*

Anti GC antisera used in C LIE or R LIE	GC antigens in the intermediate agar gel	Number of specific precipitin lines formed in C LIE or R LIE with					
		670 T2		SOA T		P9 T2	
		Op	Tr	Op	Tr	Op	Tr
670 T2 Opaque	670 T2 Opaque	–	–	–	–	–	–
»	» Transp	1[a]	–	–	–	–	–
» »	SOA T2 Opaque	1	–	–	–	–	–
» »	» Transp	1	–	–	–	–	–
» »	P9 T2 Opaque	2	1	1	1	–	–
» »	» Transp	1	1	1	1	–	–
670 T2 Transp	670 T2 Opaque	–	–	–	–	–	–
» »	Transp	–	–	–	–	–	–
P9 T2 Opaque	670 T2 Opaque	–	–	2	–	3	2
»	» Transp	–	–	2	–	3	2

a) Figure[illegible] refer to number of specific precipitin lines observed

ABLE 3 *Co agglut nation Tests of 58 Unselected Gonococcal Strains Distribution by Class W Reagents and the Occurrence of Colony Morphology Dependent Reactions*

Reagent staphylococci coated with absorbed anti GC antibodies	Tentative antigen class pattern	COA reaction pattern obtained with the 58 isolates										
Anti E 5 abs w N 10	W/I	+	+	+				c[a]				
D 4 » C 3		+			+				c[a]			
V 15 » C 3		+	+			+						
N 10 » D 4	W/II					c[a]	+	+	+	+	+	
» S 12 A 1							+	+	+	c[a]		
» F 6 » A 1 & B 2	W/III											+
Number of reacting strains		19[b]	4	1	1	1	14	1	1	1	12	3

a) c colony morphology dependent reaction
b) Colony morphology dependent reactions (Op/Tr 3 + /1 +) were obtained for three strains with one of the reagents

On the whole immunization absorption and co

) *Immunoelectrophoresis of Colour/Opac ty Colo ial Variants of Isonic Strains*

C LIE and R LIE with antigen containing intermediate gel were performed with antigen ntibody combinations of both the opaque and ransparent variants of the isogenic strains and the eterologous strains Examples of these tests are ummarized in Table 2 It will be seen that the ntigenic preparations of 670 Op had an extra ntigen not present in 670 Tr which is in agreement with the COA results Preparation of 670 and SOA shared cross reactive antigens also shown by the COA results (Table 1) The antigenic differences between P9 and 670 and SOA were confirmed in the IE tests

The antigens responsible for the specific precipi tin lines were present ...

... patterns of these were however somewhat different in C LIE tests Thus sonicated organisms gave a two peak precipitin line as previously described (3) while saline and LiCl extracts gave one-peak precipitin lines close to the antigen well These extracts gave the same reactions as whole cells in the COA test (Table 1)

3) *Distribution within Antigen Classes W and J of*

unselected gonococcal strains from male and female patients were tested with COA reagents for antigen classes W and J The W/II reagent prepared with anti U 14 absorbed with R 11 (see Table 1) was excluded since it provided no further information except subdivision of W/II The W/III reagent

... in organisms from colonies with opaque colony morphology However the previously de scribed J/6 reagent (10) prepared from anti F 6 absorbed with A 1 and B 2 did not show any cross reactivity with strains of antigen class W/II and was thus included as a new W/III reagent instead of that prepared from anti F 6 absorbed with B 2 and U 14 The overall results are shown in Tables 3 and 4

All the 58 strains could be slassified by class W reagents 26 by W/I 29 by W/II and three strains by W/III Colony morphology dependent reactions were demonstrated for four (approx 7%) of the strains three of which had the combination W/I–II or W/II–I Organisms from opaque colonies were responsible for these extra reactions Only 25 of the strains (approx 43%) ... reagents C... were found

TABLE 4 *Co-agglutination testing of 58 Unselected Gonococcal Strains Distribution by Class J Reagents and Occurrence of Colony Morphology Dependent Reactions*

Reagent staphylococci coated with absorbed anti GC antibodies	Tentative antigen class pattern	COA reaction pattern with the 58 isolates									
Anti D-4 abs w B-2 & C-3	J/4	+	c[a]					c			-
» U-14 » C-3	J/14		+	+	+						-
» X-9 » A-1 & B-2	J/9	c				+	c				-
» S-12 » X-9	J/12			+				+	+	c	-
Number of reacting strains		1	1	1	7	5	1	1	7	1	31

[a] c = colony morphology dependent reaction

from opaque colonies were responsible for four of these reactions

Two or three GC isolates were obtained from different urogenital sites (urethra, cervix or rectum) in 14 patients, making 34 isolates in all They were all tested by the class W/I and W/II and four class J reagents Identical or similar COA patterns were obtained for all the 34 isolates by the class W reagents, and for all the 16 isolates reacting with the class J reagents There were only two colony morphology dependent reactions with the class W and two with the class J reagents One isolate obtained from a patient one week after the first three positive cultures (from urethra, cervix and rectum) gave identical results as those of the previous isolates

The relatively low incidence of colony morphology dependent reactions observed with class W reagents with unselected strains was in striking contrast to the reactions obtained with class M reagents active against periodate sensitive antigens as described previously (10) A total of 14 of the 58 strains (24 %) showed colony morphology dependent reactions Ten strains (17%) gave reactions only with organisms from opaque colonies and 6 strains (10%) only with organisms from transparent colonies with at least one class M reagent Two of these strains gave reactions only with organisms from opaque colonies with some reagents, and only with organisms *from transparent* colonies with other class M reagents

When the paired isolates (34 strains) from the 14 patients were examined with the class M reagents and the colony morphology dependent reactions discussed above omitted, differences were noted for most reagents with strains *from* different sites of the same patient Eight of the 14 patients had strains that differed in the same way with organisms from both opaque and transpar colonies in the isolates from different sites of t same patient

DISCUSSION

The previously described serological classification *Neisseria gonorrhoeae* by co-agglutination (10) w confirmed in the present study We could also sho that immunization of rabbits with organis classified in this way gave antibodies whi produced the expected results when used f preparation of COA reagents We found, howev that the colour/opacity variants of isogenic T colonies, recently described by *Swanson* (11, 12) opaque and transparent respectively were of impo tance when used *for* both *immunization* and fo absorption and agglutination Organisms fro opaque T2 colonies gave extra antibodies compared to those from transparent colonies we usually more efficient for absorption, and contain an extra pronase sensitive antigen factor(s) Th was *true* for the *antigen* classes *tentatively* designa ted W and J

These findings are of interest, since *Walstad et a* (13) and *Swanson* (12) have shown that G organisms from opaque T2 colonies of isogen strains contain extra proteins as demonstrated b polyacrylamide gel *electrophoresis of outer* mem brane preparations They do not seem to b associated with pili, since they were absent in pilate transparent colonies of the isogenic strain *Walsta et al* (13) and *Swanson* (12) also showed that the were not destroyed by heat at 100 °C This is agreement with our results, since the *extra* CO reactions of these organisms were dem with heated (100 °C for 30–60 min)

t is noteworthy that the use of either transparent opaque organisms was of minor importance in paration of COA reagents for the tentatively ned antigen class W while it was of major portance in preparation of a reagent typical for tigen class J We have shown previously that .h reagents could be produced by absorption of tibodies with strains with a close antigenic atedness (10) This was confirmed in the present ork for example by absorption of anti 670 Op th organisms of SOA which were of the same W tigen class as 670 However antigen class J could demonstrated only with organisms from opaque lonies of the isogenic strains These reactions re also found to be sensitive to pronase which we d previously shown was typical for some class J actions

Walstad et al (13) and *Swanson* (11 12) recently stulated that organisms of dark-coloured i e aque colonies might have antigenic characteris s other than those of light-coloured transparent ganisms of isogenic strains In the present work e could demonstrate both by co-agglutination and nmunoelectrophoresis that organisms from opa ue T2 colonies had extra antigen(s) as compared to ose of transparent colonies of isogenic strains We ave also found that the absorbing capacity of rganisms of T3 or T4 colonies is lower than that of paque or transparent T2 colonies (unpublished bservations) Our findings might explain some of antigenic variations demonstrated by *Wilson*)

he feasibility of the co-agglutination method for ological classification of *Neisseria gonorrhoeae* orted in previous works (3 9 10) was confirmed he present study Thus all 58 GC isolates from patients could be classified by COA reagents for previously described antigen class W (10) while

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ogenital and/or rectal sites in the same patient is is in agreement with the findings reported in a vious work of repeated isolates of β-lactamase oducing gonococci from the same patient (10) It s also of interest to note that the incidence of lony morphology dependent reactions was low ith reagents for antigen class W but at least three four times as high with available reagents for tigen class J when unselected GC strains were sted It was also found that organisms from paque colonies were responsible for almost all tra reactions This was in striking contrast to the agents for antigen class M (10) All strains were ypeable but tests with these reagents showed that , from both opaque and transparent colonies of isogenic strains were responsible for variable reactions The reasons for this are not yet clear, but they might be due to the cloning of colonies with different class M antigens rather than an effect of colony morphology This was supported by the frequent finding of strains from different sites in the same patient with identical and stable class W but differing class M patterns Further investigations are needed to elucidate these matters

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TABLE 4 *Co-agglutination testing of 58 Unselected Gonococcal Strains Distribution by Class J Reagents and Occurrence of Colony Morphology Dependent Reactions*

Reagent staphylococci coated with absorbed anti GC antibodies	Tentative antigen class pattern	COA reaction pattern with the 58 isolates									
Anti D-4 abs w B-2 & C-3	J/4	+	c[a]					c			-
» U-14 » C-3	J/14		+	+	+						-
» X-9 » A-1 & B-2	J/9	c				+	c				-
» S-12 » X-9	J/12			+				+	+	c	-
Number of reacting strains		1	1	1	7	5	1	1	7	1	[illegible]

[a] c = colony morphology dependent reaction

from opaque colonies were responsible for four of these reactions

Two or three GC isolates were obtained from different urogenital sites (urethra, cervix or rectum) in 14 patients, making 34 isolates in all They were all tested by the class W/I and W/II and four class J reagents Identical or similar COA patterns were obtained for all the 34 isolates by the class W reagents, and for all the 16 isolates reacting with the class J reagents There were only two colony morphology dependent reactions with the class W and two with the class J reagents One isolate obtained from a patient one week after the first three positive cultures (from urethra, cervix and rectum) gave identical results as those of the previous isolates

The relatively low incidence of colony morphology dependent reactions observed with class W reagents with unselected strains was in striking contrast to the reactions obtained with class M reagents active against periodate sensitive antigens as described previously (10) A total of 14 of the 58 strains (24 %) showed colony morphology dependent reactions Ten strains (17%) gave reactions only with organisms from opaque colonies and 6 strains (10%) only with organisms from transparent colonies with at least one class M reagent Two of these strains gave reactions only with organisms from opaque colonies with some reagents, and only with organisms from transparent colonies with other class M reagents

When the paired isolates (34 strains) from the 14 patients were examined with the class M reagents ... dependent reactions ... were noted for most reagents ... different sites of the same patient Eight of the 14 patients had strains that differed in the same way with organisms from both opaque and transpar... colonies in the isolates from different sites of ... same patient

DISCUSSION

The previously described serological classificatio... *Neisseria gonorrhoeae* by co-agglutination (10) ... confirmed in the present study We could also sh... that immunization of rabbits with organis... classified in this way gave antibodies wh... produced the expected results when used ... preparation of COA reagents We found, howe... that the colour/opacity variants of isogenic ... colonies, recently described by *Swanson* (11, 12) ... opaque and transparent respectively were of imp... tance when used for both immunization and ... absorption and agglutination Organisms fro... opaque T2 colonies gave extra antibodies ... compared to those from transparent colonies we... usually more efficient for absorption, and contain... an extra pronase sensitive antigen factor(s) Th... was true for the antigen classes tentatively desig... ted W and J

These findings are of interest, since *Walstad et* ... (13) and *Swanson* (12) have shown that G... organisms from opaque T2 colonies of isoge... strains contain extra proteins as demonstrated ... polyacrylamide gel electrophoresis of outer mem... brane preparations They do not seem to ... associated with pili, since they were absent in pilat... ... were not des... by ... agreement with our results, since the extra CO... reactions of these organisms were demonstrat... with heated (100 °C for 30–60 min) wh... ...

IMMUNOPEROXIDASE AND ELECTRON MICROSCOPY STUDIES OF STAPHYLOCOCCAL LIPOTEICHOIC ACID

PER AASJORD and ARNE GROV*

The Gade Institute Department of Microbiology University of Bergen Bergen Norway

Aasjord P & Grov A Immunoperoxidase and electron microscopy studies of staphylococcal lipoteichoic acid Acta path microbiol scand Sect B 88 47-52 1980

Immunoperoxidase technique together with electron microscopy shows that lipoteichoic acid (LTA) of *Staphylococcus aureus* Cowan I is attached to the membrane and penetrates the whole cell wall A diffuse zone outside and no peroxidase reaction product inside the wall when whole cells were treated with antibody prior to embedding may indicate (i) that LTA is exposed to reaction with antibodies outside the wall and (ii) that all or most of the anti LTA antibodies used are of the IgM class and thus unable to penetrate the wall Thin sections of strain Wood 46 showed the same picture as Cowan I but treatment of whole cells before embedding gave no diffuse zone outside the wall This may be due to a thicker wall as found by electron microscopy and/or shorter LTA-chains of strain Wood 46 than those present in the wall of Cowan I

Key words Staphylococcal lipoteichoic acid immunoperoxidase electron microscopy

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ceived 30 vi 79 Accepted 4 ix 79

In two previous papers (19 20) staphylococcal toplasmic membrane and lipoteichoic acid (LTA) re isolated and examined chemically and serologi lly LTA was found to constitute only a minor rt of the isolated membrane Considering the embrane as an attachment site for LTA this ructure has been referred to as membrane teichoic id (2 10 13 15) There are however observa ons indicating that LTA penetrates the cell wall id depending on the length of the glycerolphos iate backbone is exposed to the surface of the cteria (4 5 18) The immunoperoxidase method r electron microscopy (1 8) is utilized in the resent study to localize LTA of *Staphylococcus ureus* strains Cowan I and Wood 46 in order to ecide how LTA is related to the cell wall

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* Deceased June 26th 1979

MATERIALS AND METHODS

Strains

Staphylococcus aureus strain Cowan I (NCTC 8530) was used for the preparation of protoplasts and LTA (19) *Staphylococcus aureus* strain Wood 46 (NCTC 10344) being protein A negative was used as a control cell and source for polysaccharide A (poly A)

Growth of Bacteria

Blood agar nutrient broth and protoplast medium were prepared as previously described (19)

Cultivation of *S aureus* strains Cowan I and Wood 46 and isolation of protoplasts by incubating bacteria in sucrose medium to which was added 1000 I U of *penicillin* was carried out as earlier (19)

Antigen Preparations

Protein A poly A and LTA were prepared as described in (7) (9) and (19) respectively

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Antigen Preparations

Protein A poly A and LTA were prepared as described in (7) (9) and (19) respectively

Sera

Antisera to LTA were produced in New Zealand White rabbits of the Institute's breed by injecting a mixture of LTA and Freund's complete adjuvant (Difco, USA) into one of the rabbit's hind foot pads, followed by four injections given intramuscularly of a mixture of LTA and Freund's incomplete adjuvant (Difco, USA) Each dose contained 2 mg of antigen and was given in intervals of two weeks

The anti-LTA serum used in the immunoperoxidase technique was absorbed on a Sepharose protein A column (14) to remove protein A reactive immunoglobulins A peroxidase labelled anti-rabbit IgG (heavy and light chain) produced in swine (DAKO-Immunoglobulins, Copenhagen, Code P2190) was used for the immunoperoxidase studies

Serum Tests

The peroxidase labelled serum and the anti-LTA serum were diluted two-fold and tested with bacterial cells by slide agglutination Subagglutinating concentrations of the sera were used in the experiments

Anti-LTA was tested for reaction with protein A and poly A by agar precipitation

Embedding Medium

Spurr (epoxy) medium was obtained from Taab Laboratories, England (16)

H_2O_2-Diaminobenzidine solutions (6, 8)

1) For treatment prior to embedding
   75 mg 3,3-diaminobenzidine-tetrahydrochloride (Fluka AG, Buchs Switzerland) in 100 ml 0 05 M Tris HCl buffer, pH 7 6, containing 0 01 per cent H_2O_2
2) [illegible]

'' Tris-HCl

Chemicals

Sodium cacodylate EM, and glutaraldehyde EM were purchased from Taab Laboratories

Sepharose 4B for the preparation of the protein A column (14) was obtained from Pharmacia Fine Chemicals AB Uppsala Sweden

Peroxidase Labelling of Bacteria and Protoplasts Prior to Embedding (8)

Cells were incubated 1 h at room temperature in antiserum against LTA diluted 1 2 with TBS (0 05 M Tris buffered saline pH 7 6 containing 0 25 per cent $MgSO_4$ and 0 02 per cent sodium azide)

After washing four times with TBS peroxidase-conjugated swine-anti-rabbit IgG diluted 1 30 in TBS, was added to the cells and incubated at room temperature for 10 min, followed by washing four times with TBS The cells were then fixed with 1 per cent glutaraldehyde in 0 1 M phosphate buffer, pH 7 2 for 20 min at 4 °C, followed by thorough washing (3 × 1 h) in phosphate buffer The bacteria were transferred to the H_2O_2 diaminobenzidine solution 1, incubated for 10 min at room temperature and washed four times with the buffer

The further treatment of the bacteria with OsO_4 dehydration was carried out using the method Kellenberger et al (11) Embedding took place in Spurr medium (16)

Protoplasts had to be fixed for 20 min in 0 1 phosphate buffer, pH 7 2, containing 1 per c glutaraldehyde, 1 6 M sucrose and 0 25 per cent MgS before further treatment The subsequent steps took p in media without sucrose as for whole cells Finally samples were cut into thin sections of about 6 thickness (estimated by reflection of light) with ultramicrotome (LKB-Produkter, Sweden) and transferred to grids (150 mesh, formvar coated) The grids treated with 2 per cent aqueous uranyl acetate for min, thoroughly rinsed in water, and then with R lead citrate for 15 min and washed in water

All electron microscopy was carried out on a H HU 12A transmission electron microscope (TEM) at 8 kV

Peroxidase-Labelling of Thin Sections of Bacteria & Protoplasts (8)

After fixation in 1 per cent glutaraldehyde in 0 1 cacodylate buffer, pH 7 4 (20 min, 4 °C), and wash with the buffer (3 × 1 h), the cells were dehydrated embedded in the Spurr medium, cut into thin sect and transferred to grids as described earlier The Sp medium of the thin sections was etched by floating grids on drops of fresh aqueous 10 per cent H_2O_2 for min and then washed three times in beakers of distil deionized water The grids were subsequently incub on drops of anti-LTA serum and peroxidase-conjug

[illegible]

the solution while stirring Anti LTA serum peroxidase-conjugated serum were diluted in TBS 1 and 1 30 respectively

Treatment with uranyl acetate and Reynolds le citrate prior to microscopy was performed as describ earlier

Except for the fixation performed in 0 1 M cacodyl buffer containing 1 6 M sucrose and 0 25 per ce $MgSO_4$ protoplasts were treated like the norm bacteria

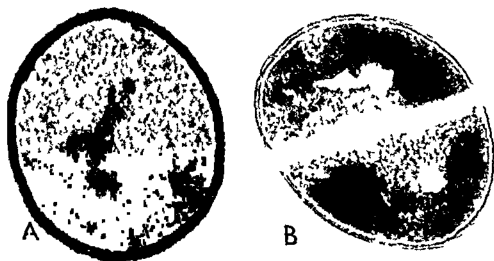
RESULTS

The electron micrographs of protoplasts showed dark narrow line covering the membrane wh protoplasts were labelled prior to embedding (F 1A) Control cells not treated with anti-LTA ser do not show this dark line (Fig 1B)

When thin sections of *S aureus* Cowan I, w treated with anti-LTA serum and peroxid conjugated serum, peroxidase reaction prod covered the whole of the cell wall in addition to t membrane (Fig 2A) In the absence of anti LT serum, no reaction product occurred on the th sections (Fig 2B) [illegible]



1 A Electron micrograph of a *S aureus* Cowan I protoplast treated with anti LTA and peroxidase-conjugated
u rabbit IgG prior to embedd ng Peroxidase reaction product is seen to be present on the protoplast membrane
) × 30 000 B Protoplast from *S aureus* Cowan I not treated with anti LTA serum × 30 000



2 A Electron micrograph of a th n section of a *S aureus* Cowan I cell treated with anti LTA and peroxidase
njugated anti rabb t IgG Perox dase reaction product seems to be present both throughout the cell wall and on the
embrane × 80 000 B Thin section of a *S aureus* Cowan I cell not treated with anti LTA serum × 80 000

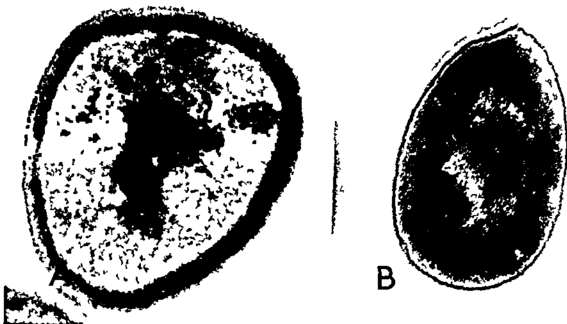


Fig 3 A Electron micrograph of a thin section of a *S aureus* Wood 46 cell treated with anti LTA and pero conjugated anti rabbit IgG Peroxidase reaction product seems to be present both throughout the cell wall and membrane × 80 000 B Thin section of a *S aureus* Wood 46 cell not treated with anti LTA serum × 8(

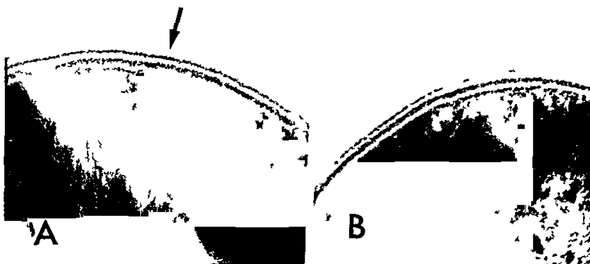


Fig 4 A Electron micrograph of a *S aureus* Cowan I cell treated with anti LTA and peroxidase-conjugated rabbit IgG prior to embedding Notice the dark zone of peroxidase reaction product outside the cell wall (ar which may be due to reaction with IgM immunoglobulins × 100 000 B *S aureus* Cowan I not treated with LTA serum × 100 000

od 46) gave the same result (Fig 3) indicating s reaction between the LTA of Cowan I and od 46

Cowan I cells treated with anti LTA serum before embedding showed a diffuse zone outside the wall (Fig 4A) in contrast to the control without LTA (Fig 4B) indicating that antibodies were and to the bacterial surface Wood 46 cells ated in the same way did not show any oxidase reaction corresponding to an anti LTA ding

DISCUSSION

The dark line which appeared outside the protoplasts after reaction with specific antibodies (anti A) and peroxidase labelled anti rabbit IgG (see 1A) confirms that LTA is attached to the mbrane Labelling of protoplasts on thin sections s not successful probably because LTA is easily eased from the membrane by washing during paration of the samples

The experiments with labelling of thin sections of aureus Cowan I showed further that LTA rastructurally is located both in the cytoplasmic mbrane and in the cell wall (see Fig 2A) Also in n sections of strain Wood 46 the entire cell wall owed reaction (see Fig 3A) demonstrating cross action between LTA of the two strains and a nilar pattern of occurrence The serum employed d no protein A or polysaccharide A reactivity No glutination of whole cells was observed (1 2 ution of serum) thus indicating the absence of glutinins

When whole Cowan I cells were treated with ti LTA serum prior to embedding there was no action on the cell wall or membrane but a diffuse ne appeared around the external surface of the all (see Fig 4A) This may be due to antibodies ainst LTA being of the IgM class (3 17) and thus their size unable to penetrate the cell wall es of the IgG class have previously been hown to penetrate the wall (8) The diffuse zone outside the wall of strain Cowan I thus indicates that part of the LTA reaches the surface of the cell Strain Wood 46 failed to give such a diffuse zone he LTA molecule is reported to have a variable number of subunits in bacteria giving rise to

[illegible]

The micrographs indicate that the Wood 46 cell wall is thicker than that of Cowan I (see Fig 2A 3A) and this may be the reason why Wood 46 cells all

That IgM antibodies are involved in the anti LTA activity is also indicated by a weaker reaction than normal for IgG antibodies (8) This may be because the peroxidase labelled antibodies against IgG only react with the light chains of IgM Furthermore involvement of IgM antibodies in the anti LTA response is discussed in (21)

The presence of LTA in the wall of *S aureus* strains and the different degree of penetration are observations which must be considered in studies on cell wall antigens

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immunoglobulins (8 14) was applied to a Sephadex G 200 column (3 × 45 cm) and eluted with phosphate buffered saline (PBS) pH 7 2 containing 0 02 per cent sodium azide The eluate was collected in 2 ml fractions at a flow rate of 6 ml per h Fractions assumed to contain the same immunoglobulins were pooled concentrated to a volume of 2 ml in collodion bags (Sartorius Membran filter Gottingen) and tested for reaction with LTA using countercurrent immunoelectrophoresis (1)

To identify the immunoglobulin classes the pooled fractions were tested with goat anti rabbit IgG (γ specific) and sheep anti rabbit IgM (μ specific) by double diffusion in agar gel Fractions were also used in the immunoperoxidase experiments

Countercurrent Immunoelectrophoresis (I) of the Anti LTA Fractions

The electrophoresis was carried out for 1 h on a 10 × 10 cm glass plate coated with 1 per cent agarose in 0 05 M veronal buffer pH 8 6 at 10 Volt per cm

Peroxidase Labelling of Thin Sections of Bacteria (5)

The bacteria *S aureus* strains Cowan I and Wood 46 were fixed with glutaraldehyde dehydrated embedded in the Spurr epoxy medium and cut into thin sections with an ultramicrotome (LKB Produkter Sweden) (see 14)

The labelling took place by incubation of H_2O_2 etched thin sections with the pooled anti LTA fractions followed by incubation with peroxidase conjugated anti rabbit IgG and reaction with the diaminobenzidine solution The procedure was as described previously (5 14)

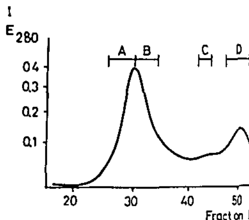
Electron microscopy was carried out on a Hitachi HU 12A transmission electron microscope (TEM) at 100 kV

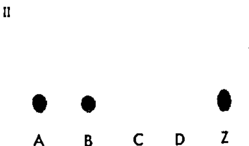
RESULTS

Two main immunoglobulin peaks and a small intermediate one appeared on fractionation of the rabbit anti LTA serum on the Sephadex G 200 column (Fig 1 I) Countercurrent immunoelectrophoresis showed that the fractions A and B as well as unfractionated anti LTA serum precipitated with LTA (Fig 1 II) whereas fractions C and D did not The protein A reactive immunoglobulins bound to the Sepharose protein A column gave on testing no precipitation line with LTA

Double diffusion in agar showed that the fractions A and B contained the IgM class of immunoglobulins only and D the IgG class (Fig 1 III) Fraction C contained IgG and most probably also IgA but in too low concentrations to be observed in double diffusion Fraction B from the IgM peak and the fractions C and D were further used in the immunoperoxidase experiments

Labelling thin sections of bacteria with the separated fractions showed that only those sections





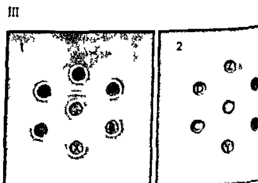
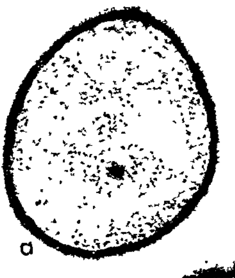


Fig 1 I Fractionation of anti LTA serum on Se G 200 A and B mark the IgM peak D the IgG pe fractions from region C contained IgG and pr some IgA Fractions were collected as indicated bars

II Countercurrent immunoelectrophoresis of th LTA fractions in 1 per cent agarose for 1 h in 0 veronal buffer pH 8 6 10 Volt per cm then co with Coomassie Brilliant Blue R (Sigma USA upper wells contain LTA (1 mg/ml) and the lower the serum fractions (A B C D) and unfractionate LTA serum (Z)

III Fractions A B C and D tested against goa rabbit IgG (γ specific) (1) and sheep anti rabbit Ig specific) (2) in agar precipitation Glass plates coate 1 2 per cent water agar were used The test sera



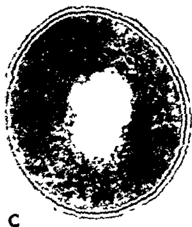




Fig 2 Electron micrographs of immunoperoxidase treated thin sections of *S aureus* strain Cowan I (a) and strain Wood 46 (b) treated with the IgM fraction from Sephadex G 200 Peroxidase reaction product is seen on the cell wall of both bacteria Treatment of thin sections of Cowan I cells with the IgG fraction (c) gave no peroxidase reaction on the cell wall Magnification 80 000 ×

hich were treated with the fraction containing M did show the presence of peroxidase reaction roduct (*Fig 2a and b*) Treatment of thin sections ith the fractions C and D containing IgG (and robably IgA as well in C) showed no reaction Nor as any reaction observed by treatment of thin ections with the peroxidase-conjugated serum lone

All four anti LTA sera used in the experiments

DISCUSSION

Fractionation of the rabbit anti LTA serum followed by countercurrent immunoelectrophoresis precipitation in agar and immunoperoxidase technique applied to bacterial thin sections clearly showed that IgM is the immunoglobulin which interacts with LTA The anti LTA serum was depleted of protein A reactive immunoglobulins and did not react with the wall teichoic acid (poly A)

The IgM peak was collected in two separate parts (A and B) This was done to avoid a possible interference of other serum proteins or complexes which may be present in fraction A However fractions A and B gave identical results in the experiments

The results are in accordance with other observations that the specific IgM antibodies are unable to penetrate the cell wall in contrast to IgG (5) Staining is relatively weak due to peroxidase labelled anti rabbit IgG (heavy and light chain) Thus in the present experiments only reactions with light chains of the IgM antibodies occurs

Anti LTA antibodies of the IgM class are previously reported by *Wicken & Knox* (11)

especially antibodies directed against sugar moieties of the LTA Immunoglobulins of the IgG class are reported to be directed against the glycerophosphate backbone (11) In the present experiments no anti-LTA activity was observed in the IgG fraction Gas liquid chromatography showed that the extraction procedure used to isolate LTA from *S aureus* Cowan I leaves the molecule intact (12) Thus, if antibodies against the glycerophosphate backbone are of the IgG class, no antibodies of this specificity are present The degree of sugar substitution has been shown to influence the antibody response to the polyol phosphate backbone (7) Furthermore, the animal species, the vaccine composition and the route of administration selected are important factors in this connection

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BRIEF REPORT

LACK OF EVIDENCE FOR AN ASSOCIATION BETWEEN INFECTION WITH *CHLAMYDIA TRACHOMATIS* AND CROHN'S DISEASE, AS INDICATED BY MICRO-IMMUNOFLUORESCENCE ANTIBODY TESTS

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Mårdh P A Ursing B & Sandgren E Lack of evidence for an association between infection with *Chlamydia trachomatis* and Crohn s disease as indicated by micro-immunofluorescence antibody tests Acta path microbiol scand Sect B *88* 57–59 1980

A significant difference has recently been reported in the occurrence of serum antibodies to the lymphogranuloma venereum (LGV) immunotypes of *Chlamydia trachomatis* in patients with Crohn s disease and controls In the present study sera from 107 patients with Crohn s disease 33 males and 74 females were tested for antibodies to *C trachomatis* by an indirect immunofluorescence test, using two pools of antigens These pools contained antigens to immunotypes TRIC D K and LGV 1–3 respectively None of the patients had IgM antibodies to the organism IgG antibodies occurred significantly more often in the female than in male patients No correlation could be demonstrated between activity and duration of Crohn s disease and the results of the antibody tests For comparison the sera of 50 puerperal women were studied No difference in the occurrence of IgG antibodies was found between patients and controls neither when using the two pools of antigen thus indicating that the antibodies detected had probably been induced by TRIC rather than LGV organisms To conclude the study did not indicate an association between Crohn s disease and infection with *C trachomatis*

Key words Crohn s disease *Chlamydia trachomatis* serum antibodies LGV TRIC

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The aetiology of Crohn s disease the frequency of hich seems on the increase (10) is unknown A variety organisms including anaerobic bacteria mycobacteria d viruses have been considered as possible aetiological ents (12) Recently *Schuller and coworkers* (5) in the S reported that they found antibodies to *Chlamydia rachomatis* of the lymphogranuloma venereum (LGV) erotypes in 38 of 55 patients with Crohn s disease but ignificantly less often in patients with other gastrointes inal disorders and in healthy blood donors Frei s test as been reported positive in patients with Crohn s disease (8)

In calves *Chlamydia psittaci* may cause epithelioid granulomas (1 4) The possible occurrence of gastroin estinal infections with *C trachomatis* viz. the other xclusively human species of *Chlamydia* has so far been little explored

One hundred and seven patients with Crohn s disease 33 males and 74 females were studied They were selected at random among the approximately 300 patients with this disease at present under treatment at the Department of Infectious Diseases University Hospital *Lund Sweden* The diagnostic criteria for Crohn's disease used were the same as those presented *elsewhere (3)* Differentiation of the clinical material into active and non active cases was based on a combination of clinical and laboratory parameters Although consider able *difficulties* are encountered in attempting to determine the duration of disease in a given case the material was divided into cases where there had been symptoms suggestive of Crohn s disease for three years of longer and those with symptoms of shorter duration Fifty puerperal women from the same hospital catch ment region served as controls

Sera from the patients were tested for IgM and IgG

TABLE *IgG Antibodies to* Chlamydia trachomatis *in Patients with Crohn s Disease in Relat on to Sex of Pa en Duration and Activity of Disease and in Puerperal Control Women*

Group	Sex	Duration (yrs) and activity of disease		No of patients studied	Titre of IgG antibodies to *C trachomat s*					
					≤16	32	64	128	256	>'
Patients	males	<3	active	0	0	0	0	0	0	[illegible]
			inactive	6	5	1	0	0	0	[illegible]
		>3	active	6	6	0	0	0	0	[illegible]
			inactive	21	19	2	0	0	0	[illegible]
	females	<3	active	4	2	0	0	2	0	[illegible]
			inactive	16	12	3	1	0	0	[illegible]
		>3	active	10	8	2	0	0	0	[illegible]
			inactive	44	31	4	5	3	1	[illegible]
Controls	females	–		50	38	6	1	0	2	[illegible]

antibodies to *C trachomatis* by a micro immunofluore scence test (9) using two pools of antigen One pool consisted of antigens of *C trachomatis* immunotypes TRIC (trachoma inclusion-conjunctivitis agents) D K and the other of antigens of immunotypes LGV 1–3

The occurrence of IgG antibodies to *C trachomatis* reacting with LGV 1–3 is shown in the Table None of the patients had such IgM antibodies IgG antibodies to *C trachomatis* occurred significantly more often in the female than in the male patients No relationship was found between the presence of antibodies and the duration and activity of Crohn s disease Of the controls 16% had IgG antibodies to *C trachomatis* with a titre of ≥64 while the corresponding figure in the female patients was 12%

With a few exceptions testing the sera by using the [illegible]

among the sera containing antibodies to *C trachomatis* and never exceeded one dilution step

Our findings of antibodies to *C trachomatis* in patients with Crohn s disease are not in principle in accordance with those of *Schuller et al* (5) but are similar to those of two recent reports from the U K (6 [illegible] material [illegible] ctivity of [illegible] Crohn s disease from the point of view of [illegible] arameters did not indicate that the occurrence of serum antibodies to *C trachomatis* was associated with acute stage Crohn s disease Neither did the lack of IgM antib to this organism suggest acute infections with *trachomatis* as being associated with that disease

The finding that antibodies to *C trachoma s* [illegible] demonstrated significantly more often in female th male patients with Crohn s disease is in conform [illegible] studies on patients with no evidence of that d sease The similarity of the result in the tests using the different pools of antigens suggests that the antib demonstrated in the sera of the patients and controls probably been produced against TRIC organ Antibodies to these organisms cross react broadly LGV antigens (11)

The study was supported by grant 16X 04509 from Swedish Medical Research Council

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a path microbiol scand Sect. B 88 61–63 1980

BRIEF REPORT

GROWTH INHIBITION OF *STREPTOCOCCUS MUTANS* STRAIN OMZ 176 BY XYLITOL

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Assev S Vegarud G & Rolla G Growth inhibition of *Streptococcus mutans* strain OMZ 176 by xylitol Acta path microbiol scand Sect. B 88 61–63 1980

The present study showed that xylitol exhibited a dose related inhibition of the growth of *Streptococcus mutans* in a Brain Heart Infusion medium It is suggested that the mechanism involved may be an effect of the translocation of glucose across the bacterial cell membrane Sorbitol showed no similar effect but caused a delay in the reaching of the stationary phase

Key words Xylitol sucrose substitutes dental caries

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Xylitol and sorbitol are used as sucrose substitutes in eral sweets to improve dental health because of the iogenicity of sucrose particularly when consumed tween meals There are controversial reports in the rature concerning the effect of xylitol on the growth of *eptococcus mutans* which is thought to be the primary iological agent of dental caries *Makinen* (1972) owed that the growth of strain Ingbritt was inhibited xylitol but reported normal growth of this strain 4–5 onths after the continuous presence of xylitol in the ltures Growth inhibition was also reported by *avenaar et al* (1978) whereas *Muhlemann et al* (1977) ere unable to show any such effect in strain OMZ 176 ne present study was initiated to compare the effect on e growth of *Strep mutans* strain OMZ 176 of the idition of xylitol or sorbitol to the culture media

laterial and Methods

Bacteria Streptococcus mutans strain OMZ 176 was btained from Dr *B Guggenheim* Zurich

Growth medium Bacto Brain Heart Infusion (BHI) ontaining 0 16% glucose was used as a basic medium

Carbohydrates D Sorbitol and Xylitol were purcha ed from Fluka AB and D (+) Glucose (anhydrous) rom *Merck*

Growth measurement The growth of the cultures was neasured at 650 nm against water in a Zeiss P M Q II ...ment

Growth inhibition experiments 5 ml of a stationary phase culture was inoculated into 100 ml batches of sterilized (20 min 110 °C) BHI medium The carbohydrate solutions were sterilized separately and added to the medium in order to obtain the concentration specified below The cultures were then incubated at 37 °C

1 ml aliquots were drawn at zero time and at every hour during the log phase and every second hour during the stationary phase The last aliquots were taken 26 hours after inoculation *Strep mutans* strain OMZ 176 was grown in BHI with either no sugar added with 2 5% glucose added or with 1% 4% or 5% sorbitol or xylitol added

Each series of experiments was carried out simultaneously with medium from the same batch and carbohydrates from the same solutions

Results

The first series of experiments showed that 5% of xylitol in the medium inhibited growth compared with the basic BHI medium with no extra carbon source added Addition of both xylitol and glucose to the cultures gave a reduction in growth as compared to addition of glucose alone and the inhibition was dose related (Fig 1)

Similar experiments with sorbitol did not show such clear effects Addition of 5% sorbitol to the basic medium gave a slightly higher growth after 24 hours although the stationary phase of growth was reached

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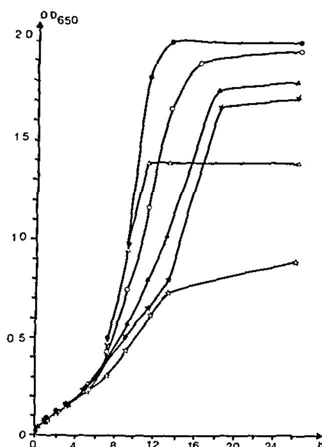


Fig 1 *Strep mutans* strain OMZ 176 grown in BHI with glucose and xylitol ☆———☆ 5% xylitol △———△ no carbohydrates ●———● 2 5% glucose ○———○ 2 5% glucose and 1% xylitol ▲———▲ 2 5% glucose and 4% xylitol ✶———✶ 2 5% glucose and 5% xylitol

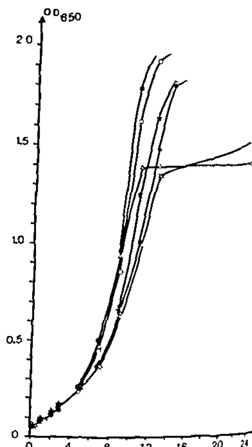


Fig 2 *Strep mutans* strain OMZ 176 grown in BHI with glucose and sorbitol ☆———☆ 5% sorbitol △———△ no carbohydrates ●———● 2 5% glucose ○———○ 2 5% glucose and 1% sorbitol ▲———▲ 2 5% glucose and 4% sorbitol ✶———✶ 2 5% glucose and 5% sorbitol

later in the sorbitol culture Addition of 1% sorbitol to a culture containing 2 5% glucose showed no effect on the final growth level The addition of 4% and 5% of sorbitol had an inhibitory effect though this was less marked than with xylitol (Fig 2)

Discussion

Several reports have indicated that *Strep mutans* is unable to metabolize xylitol whereas sorbitol is metabolized slowly by this microorganism as measured by acid production in cultures (*Havenaar et al* 1978) Our experiments supported the observations of *Makinen* (1972) and *Havenaar et al* (1978) in demonstrating a reduction by xylitol on the growth of different strains of *Strep mutans* This was particularly apparent when no other carbon source was added but could also be observed with glucose and the effect was dose related

Sorbitol did not show any similar effect in the experiment where no glucose was added However the stationary phase was reached later but the level of growth was higher after 24 hours in the sorbitol cultures thus indicating some metabolization of this carbon source by *Strep mutans* Addition of sorbitol to cultures containing extra glucose showed some reduc[tion in] growth and a longer time used in reaching the stat[ionary] phase but this was not as marked as in [the] experiments with xylitol

The mechanism of the inhibition described ab[ove is] unknown It seems conceivable that an effect [on the] translocation of glucose across the bacterial cell [mem]brane occurs either on the phosphotransferase syste[m or] permease system or through blocking of [the] sugarbinding proteins (*Makinen* 1978) Xylitol is [known] to be a potent inhibitor of the D Xylose isomer[ase in] *Lactobacillus brevis* (*Yamanaka* 1969) It is not [known] whether or not the growth reducing effect of the[se] substitutes may be of clinical significance in their [use in] the oral cavity but this seems possible

Recent research has shown that dental plaque f[ormed] in the presence of xylitol exhibits changed ph[ysical] properties as compared to plaque formed in the pr[esence] of sucrose the former being less adhesive an[d more] hydrated It also contains less glucan and lipote[ichoic] acids (*Rolla et al* 1980) Growth inhibition of [*Strep*] *mutans* by xylitol could conceivably be of significa[nce in] this respect in reducing the metabolic processes occ[urring] in dental plaque No similar data are available c[...]

BRIEF REPORT

ENTEROTOXIN PRODUCTION BY *YERSINIA ENTEROCOLITICA* AND *YERSINIA ENTEROCOLITICA* LIKE MICROBES AT 22 °C and 37 °C

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Kapperud G Berdal B P & Omland T Enterotoxin production by *Yersinia enterocolitica* and *Yersinia enterocolitica* like microbes at 22 °C and 37 °C Acta path microbiol scand Sect. B 88 65–67 1980

A total of 32 strains of *Yersinia enterocolitica* and *Y enterocolitica* like microbes were examined for their ability to produce *E coli* ST like enterotoxin when grown at 22 °C and 37 °C using the infant mouse assay Enterotoxin production was indicated for 19 (59%) of the 32 strains examined Nine of ten sucrose non fermenting strains belonging to O serogroup 28 produced enterotoxin at both 22 °C and at 37 °C Eight of these strains were obtained from the intestinal contents of apparently healthy small mammals or from water samples at three different localities in Norway One O serogroup 28 culture was a reference strain received from Institut Pasteur Paris With the exception of O serogroup 28 enterotoxins were detected only after incubation at 22 °C The infant mouse assay was positive for all the four clinical isolates belonging to O serogroup 3/biotype 4 Six of 13 different reference strains were positive in this assay

Key words *Yersinia enterocolitica* enterotoxin

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n previous studies we demonstrated that *Yersinia* *rocolitica* and *Y enterocolitica* like microbes are quitous in both terrestrial and freshwater ecosystems Scandinavia (2 3 4 5) A broad diversity of ologically and biochemically different types were lated However the significance of most of these ains as a cause of animal or human disease is known The epidemiology/epizootology of this kind yersiniosis is still not completely understood

In recent years interest has been focussed on the rch for pathogenicity factors among strains of *Y terocolitica* (7 8 9 11) This may represent an portant clue towards a more complete understanding the epidemiology and ecology of this microbial group i et al (8) reported that production of *E coli* ST like

In the present investigation a pilot survey comprising *Y enterocolitica* reference strains and clinical isolates indicated enterotoxin production at 37 °C by one representative of *O serogroup 28* This observation prompted us to consider a more extensive material of this O-serogroup

Materials and Methods

1 Bacterial Strains

A total of 32 cultures of *Y enterocolitica* and *Y enterocolitica* like microbes were examined Their serological properties and source of isolation are presented in *Table 1* The strains fall into three different groups

1 Reference strains A total of 13 type strains representing different O serogroups of *Y enterocolitica* were included Twelve strains were received from Institut Pasteur Paris one was obtained from Dr Winblad Malmø

2 Clinical isolates Ten clinical isolates four of which belonged to O serogroup 3/biotype 4 (Wauters biotype scheme) were examined Nine strains were isolated from

Enterotoxin Assay

Enterotoxin activity was tested using the infant mouse y (1) Sterile culture filtrate (0 1 ml) was injected ugh the abdominal wall into the milk filled stomach hree 3–4 day-old mice (NMRI(BOM)sp) Injected were kept at room temperature for four hours The imals were killed and the entire intestine was removed etermine the ratio weight of intestine to remaining y weight Ratios of ≥ 0 083 were considered tive This value is accepted as a minimum for monstration of *E coli* ST enterotoxin in the infant use assay (10)

ults

Results are presented in Table 1

he presence of *E coli* ST like enterotoxin in culture ates was indicated for 19 (59%) of the 32 strains of *sin a enterocolit ca* and *Y enterocol tica* like micro examined by infant mouse assay Encompassed by study are representatives of 16 O-serogroups n of which showed enterotoxin production

Nine of ten isolates belonging to O-serogroup 28 (suc non fermenting) produced enterotoxin when cultu at both 22 °C and at 37 °C With this exception rotoxin was demonstrable only when organisms e grown at 22 °C

All the four strains belonging to O serogroup 3/ ype 4 were positive for enterotoxin Another six ins isolated from human patients with gastrointestu d sease belonging to other O-serogroups gave ative results

Among the 13 reference strains examined enterotoxin s detected for strains representing six different O ogroups

cussion

Hitherto published investigations concerning entero in production by *Yersinia enterocolitica* have not luded O serogroup 28 This O serogroup was first lated nine years ago from untreated drinking water in Norway by *Lassen* (6) Reports of isolation from other countries are very sparse

So far no pathogenic properties have been ascribed to O serogroup 28 However this serogroup acquires a new significance from the present study where it gave the first known indication of enterotoxin production by *Y enterocolitica* at 37 °C With the exception of this serogroup enterotoxin production in *Y enterocolitica* has been found only when strains were grown at temperatures lower than those prevailing in the bodies of mammals and birds

For the present one should question the clinical significance of *Y enterocolitica* produced enterotoxin Despite this in view of the present findings the possibility of a pathogenic role of O serogroup 28 should be reconsidered

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We want to thank *Jørgen Lassen* and *Jorunn Sundar* for the clinical isolates provided The excellent technical assistance of *Merete Mathiesen* is gratefully acknowl edged

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human patients with gastrointestinal disease at the National Institute of Public Health, Oslo One strain was isolated from swine

3 Environmental strains Included are nine strains obtained from terrestrial and freshwater ecosystems in three different communities in Norway Isolations were made from the intestinal contents of apparently healthy small mammals and from water samples (3) All strains were antigenically related to O serogroup 28 and did not ferment sucrose All except one strain were Voges Proskauer negative (both at 37 °C and at room temperature) and aesculin negative Four strains were salicine negative

The procedure followed in serological and biochemi cal characterization has been specified elsewher Strains were stored as stab cultures

II Preparation of Sterile Culture Filtrates

The strains were inoculated into 5 ml broth (TSB) containing 0 6% yeast extr Broths were incubated for 48 hours w rotary shaker (200 rev/min) at room proximately 22 °C) and at 37 °C The cu centrifuged (4000 rev/min for 30 minute supernatants were filtered sterilely throug filters (pore size 0 45 µm) Filtrates were sto and examined for the presence of enteroto within one week

TABLE I *Enterotoxin Production by Clinical and Non Clinical Isolates of* Yersinia enterocolitu enterocolitica *Like Microbes Indicated by Infant Mouse Assay*

Strain no		Source	O serogroup		Enterotoxin produ 22 °C	3
96 I P	Reference strains	Chinchilla	4–32		–	
1476 I P	»	Water	4–33		–	
123 I P	»	Cow	5A		+	
885 I P	»	?	5–27		+	
102 I P	»	Human	6–30		–	
FY 50	»	?	8–19		+	
201 I P	»	Human	9		–	
867 I P	»	Human	16–29	Rh +[a]	–	
846 I P	»	Human	18		–	
845 I P	»	Human	20		+	
1367 I P	»	Human	22		+	
1474 I P	»	Water	28	S–[b]	+	
1501 I P	»	?	34		–	
9595/76	Clinical isolates	Human	3		+	
306/77	»	Human	3		+	
365/77	»	Human	3		+	
311/77	»	Swine	3		+	
6526/76	»	Human	4–32		–	
5367/76	»	Human	5		–	
5831/76	»	Human	6–30		–	
R37/77	»	Human	14		–	
9728/76	»	Human	16	Rh +	–	
R6/77	›	Human	NT[c]		–	
11051 A	Environmental strains	Shrew	28	S–	+	
9021 B		Shrew	28	S	+	
9019 A	»	Shrew	28	S–	+	
1003 A	»	Shrew	28	S–	+	
10016 B	»	Shrew	28	S–	+	
9008	»	Small rodent	28	S–	+	
1030	»	Small rodent	28	S–	+	
G2	»	Water	28	S–	+	
G13 A	»	Water	28	S–	+	

[a] Rhamnose positive
[b] Sucrose negative
[c] Not typeable

A VACUUM GADGET FOR SAFE AND RAPID MOUNTING OF COVER SLIPS ON SLIDES WITH LIVING PATHOGENIC MICROORGANISMS

ERNST RASMUSSEN

Statens Seruminstitut Treponematoses Department Copenhagen Denmark

Rasmussen E A vacuum gadget for safe and rapid mounting of cover slips on slides with living pathogenic microorganisms Acta path microbiol scand Sect B 88 69–70 1980

A simple gadget is described for the safe and rapid mounting of cover slips on slides with infectious materials The instrument has worked satisfactorily in the mounting of more than 50 000 slides

Key words Safe cover slips pathogenic microorganisms

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Mounting of cover slips on slides for microscopy olves a certain risk when working with infecus materials Furthermore it is timeconsuming pick up the cover slips and mount them carefully large numbers of slides No instructions for ilitating this process were to be found in text oks on microbiology and microscopy (1 2 4) To knowledge of the writer no suitable and simple trument is commercially available and it has n confirmed that no patents h

ni Sweden the United Kingdom or the USA (3) safe highly efficient and inexpensive vacuum dget is presented here

CONSTRUCTION AND FUNCTION

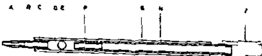


Fig 1 Construction of vacuum gadget for mounting cover slips For explanation see text

mm bore 7 mm A tube of stainless steel H length 100 mm is pushed 80 mm into the other end of G F is a tapered tube of plastic length 15 mm fitted to H The tip of F is a little uneven A steel ball E diameter 6 mm is placed into D the partition between C and F H is connected to a vacuum pump by I a flexible tube of

E is brought to F where it does not close completely This saves unnecessary suction of air and prevents noise from suction If the tip of A is blocked by a cover slip the compartment between the tips of A and F is evacuated and E is released

In operation the gadget is held like a pencil with the tip somewhat downwards A cover slip is taken by pressing the gadget gently on to the cover slip perpendicular to its surface (Fig 2a) After less than one second the cover slip can be lifted (Fig 2b) The cover slip is mounted on the slide by pressing an edge of the

a path microbiol scand Sect B 88 71-77 1980

QUANTIFICATION OF BACTERIA IN OPERATION WOUNDS – AN EXPERIMENTAL INVESTIGATION WITH SWAB SAMPLING IN PIGS

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Nystrom P-O Quantification of bacteria in operation wounds – an experimental investigation with swab sampling in pigs Acta path microbiol scand Sect B 88 71-77 1980

Quantitative recovery by swab cultures of *Staphylococcus aureus* or *Escherichia coli* from experimental wounds in pigs was studied for assessment of wound contamination in elective surgery The recovery rate declined rapidly over the first hour Ten minutes after seeding the wounds with known numbers of bacteria the recovery was respectively 0 7% and 4 1% for *S aureus* and *E coli* Recovery was not affected by the size of the wound Streaking the swab onto the culture plate or rinsing the swab in saline and plating out the rinsing fluid immediately after sampling resulted in a three to four times higher yield than by using Stuart s transport medium for later plating The results provide an indication of the discrepancy between the number of micro-organisms recovered from wounds in elective surgery and the number of micro-organisms required to produce suppurative infection in experiments on animals and in man

Key words Operation wounds bacteria quantification pigs

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eceived 20 viii 79 Accepted 21 ix 79

The risk of post-operative wound infection is lated to intra-operative contamination of the ibcutaneous wound with bacteria, as shown by the gher infection rate in patients with a positive ilture from the wound on closure (*Dillon et al* 969 *Davidson et al* 1971 *Brote* 1976)

Quantification of the bacterial population in the ound should provide information for further nalysis of the role of bacteria in the pathogenesis of ost-operative infections However little is known oncerning the accuracy and significance of quanti itive wound cultures in elective surgery though xperience is accumulating in the field (*Taylor* 1961 *Burke* 1963 *Lilly et al* 1970 *Raahave* 1974 1976 979 *Sanderson & Bentley* 1976 *Bartlett et al* 978 *Scheibel et al* 1978)

Obviously counts of bacteria from wound ultures do not represent the true number of ... reaching the wound in the course of surgery It is unlikely that all bacteria can be removed from the wound by the sampling device or can be totally transferred to the culture medium

The aim of this study was to give an experimental account of the relationship between the wound population of bacteria and the number of bacteria recovered from the wound by quantitative wound swab cultures Attempts were made to identify some of the factors which influence the recovery rate

MATERIALS AND METHODS

Pigs of the Swedish land race weighing about 20 kg were anaesthesized Tracheotomy with endotracheal intubation was performed and the normal body temperature maintained Physiological saline was infused intravenously during the experiments

The sides of the animals were shaved and the skin disinfected with 0 5% chlorhexidine in 70% alcohol

a



b



c



Fig 2 Vacuum gadget in operation picking up (a) transferring (b) and mounting (c) cover slip For details see text

slip gently on to the slide (Fig 2c) It is released from gadget as soon as the slide is not perpendicular to gadget

RESULTS AND DISCUSSION

The gadget has been used by laboratory techn for mounting more than 50 000 cover slips slides with live pathogenic bacteria The moun can be performed gently and rapidly There is risk of contamination of the operator nor any of fingers being cut

The gadget can be used after a brief instructio is durable and can be produced at a low price

Borge B Jorgensen and *J Riis Nielsen* are thanked help with preparation of the manuscript

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he results are shown in Tables 1 and 2 Mean
very was 0 7% with *S aureus* and 4 1% with
oli The differences between lowest and highest
very from single wounds within each experi
nt (ten wounds same seeding) varied from 3 fold
to 30 fold When wounds were seeded with
× 10^2 CFU of *S aureus* four out of ten wounds
e culture negative and no culture showed more
n two CFU

Recovery followed an approximately linear rela
ship with inoculum thus suggesting that wound
lture count was dependent mainly on the wound
terial population up to 10^5 CFU per wound A
ear regression analysis of y = log wound culture
int on x = log inoculum count was performed on
experiments with *S aureus* in order to assess
prediction errors involved Roughly an allo-
nce of one logarithm in either direction seemed to
involved when predicting wound culture count
m inoculum count and aiming at 95% confi
ice in prediction Inversely when predicting
und population from wound culture count, the
responding margin of error was about one and
half logarithms However available data did not

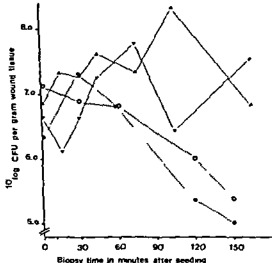
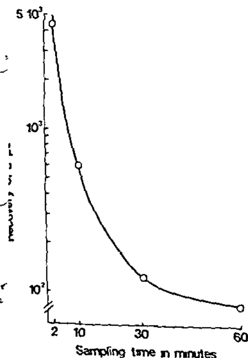


Fig 2 Change in wound tissue concentration (CFU/g) with time after seeding experimental wounds in pigs with either *Escherichia coli* (solid line) or *Staphylococcus aureus* (broken line) A decline in tissue concentration of *S aureus* was observed but not of *E coli*



g 1 Decline in recovery with time of *Staphylococcus aureus* from experimental wounds in pigs Wounds were seeded with 1 2 × 10^5 *S aureus* and sampling was performed with cotton swabs at 2 10 30 or 60 min after inoculation Each entry is the mean of ten wounds

yield good estimates of the variance components involved and in consequence the limits must be taken with caution

Effect of Time on Recovery

To determine the recovery as a function of time after inoculation forty wounds in sets of ten were seeded with 1 2 × 10^5 *S aureus* and sampled at 2 10 30 and 60 min respectively

A non linear decline in recovery was observed (Fig 1) At 2 min mean recovery was 4 4 × 10^3 CFU (3 6%) and this declined to 8 2 × 10^1 CFU (0 07%) at 60 min

Corresponding results were obtained when wounds were seeded with *E coli* Mean recovery was 4 1% at 10 min and this declined to 1 8% when sampling was performed 30 min after seeding (Table 2)

The significance of time on recovery was evaluated further by performing the following experiment Stab wounds 1 cm in length and 1 cm in depth were made and seeded by cotton swabs dipped in 10^8 CFU per ml of either *S aureus* or *E coli* which were allowed to remain in the wounds for 15 minutes The wounds were excised at various times after seeding and the tissue concentration of bacteria was determined

The results showed that recovery of *S aureus* per gram tissue declined with time after seeding On the other hand recovery of *E coli* did not decline during the first 165 min after seeding (Fig 2)

Subsequent agar skin contact cultures were negative A number of 4 cm incisions were made through skin and subcutaneous tissue Bleeding was controlled by covering the wounds with sterile gauze for five minutes

Staphylococcus aureus (Oxford strain 209, AB Bio disk) and four clinical isolates of *Escherichia coli* were used for the experiments An overnight dextrose broth culture was diluted serially in saline and the number of colony forming units (CFU) was determined by viable count performed immediately before the experiments Wounds were inoculated with 0 1 ml of bacterial suspension from one of the dilution tubes The exact amount of bacterial suspension was administered to the wounds by micropipette and spread uniformly Sampling was performed with a dry cotton swab (Torrent Corp) which was moved backwards and forwards several times in the seeded wound and then streaked in a standard manner on a 9 cm blood agar culture plate The number of CFU per plate was registered When growth was heavy two representative areas on the plate were counted and the total growth estimated as described previously (*Nystrom* 1978)

In one part of the study wound tissue concentration of bacteria was determined The entire wound was excised and the skin was separated from the subcutaneous tissue and discarded The remaining soft tissue weighed and diluted 1 10 (weight/volume) in thiogl late broth then homogenized with rotating knives homogenate was diluted serially and 0 1 ml of dilution was spread on blood agar culture plates C forming units per gram tissue were calculated t into account dilution cultured volume and weig sample

EXPERIMENTS AND RESULTS

Effect of Numbers of Seeded Bacteria on Reco

This series of experiments was intended determine the number of bacteria recovered a function of the number of bacteria inoculated to define the lower limit for detection of tion

In nine experiments with *Staphylococcus aur* and four experiments with *Escherichia coli* fr 1 2 × 10² to 1 8 × 10⁵ CFU in 0 1 ml were into the wounds Sampling was made after and the swab was streaked immediately onto culture plate

TABLE 1 *Quantitative Recovery by Swab Sampling 10 min after Inoculation of Experimental Wounds in Pigs* Staphylococcus aureus

Expt No	Inoculum per wound	No of wounds	Recovery per wound Mean ± SEM CFU	Recovery as percentage of inoculum mean and range
1	$1\ 2 \times 10^2$	10	0 8 ± 0 2	0 7 (0–1 7)
2	$1\ 2 \times 10^3$	10	18 2 ± 1 5	1 5 (0 9–2 3)
3	$1\ 8 \times 10^3$	10	13 7 ± 3 6	0 8 (0 06–1 9)
4	$1\ 0 \times 10^4$	9	172 0 ± 33 3	1 7 (0 4–3 5)
5	$1\ 2 \times 10^4$	10	82 4 ± 15 7	0 7 (0 09–1 3)
6	$1\ 8 \times 10^4$	10	27 9 ± 5 1	0 2 (0 07–0 3)
7	$1\ 2 \times 10^5$	10	423 4 ± 116 1	0 4 (0 05–1 0)
8	$1\ 2 \times 10^5$	10	587 2 ± 91 7	0 5 (0 1–1 0)
9	$1\ 8 \times 10^5$	9	248 9 ± 73 3	0 1 (0 06–0 5)

TABLE 2 *Quantitative Recovery by Swab Sampling 10 or 30 min after Inoculation of Experimental Wounds in with* Escherichia coli[a]

Expt No	Time of sampling (min)	Inoculum per wound	No of wounds	Recovery per wound Mean ± SEM CFU	Recovery as percentage inoculum mean and ra
1	10	$1\ 0 \times 10^4$	9	638 4 ± 95 5	6 4 (1 7–12 1)
2	10	$1\ 6 \times 10^4$	9	533 2 ± 59 8	3 3 (1 8–5 4)
3	10	$1\ 8 \times 10^4$	10	533 6 ± 80 1	3 0 (1 4–5 2)
4	10	$5\ 0 \times 10^4$	10	1 836 2 ± 284 3	3 7 (1 6–6 8)
5	30	$8\ 7 \times 10^3$	10	142 5 ± 20 0	1 6 (1 1–2 9)
6	30	$1\ 1 \times 10^5$	8	2 022 0 ± 361 2	1 8 (0 6–3 2)
7	30	$1\ 1 \times 10^5$	10	2 338 6 ± 240 8	2 1 (1 1–3 5)

[a] Four clinical isolates of *E coli* were used in the experiments

3BLF 4 *Relative Eff cacv of Three D fferent Swab Handling Procedures for Quantitative Recovery of* Staphylococcus aureus *from Experimental Wounds in Pigs*

Expt No	Inoculum per wound	No of wounds	Swab procedures Stuart[a]	Immediate[b]	Rinse[c]
1	1 8 × 10^3	10	1	4 5	7 0
2	1 8 × 10^4	10	1	1 5	2 0
3	1 2 × 10^5	10	1	3 6	4 1
4	1 8 × 10^5	9	1	2 8	3 5
		Mean	1	2 9	4 2

Swab stored for 18–24 hours in Stuart transport medium before streaking swab *on culture medium*
—Streaking of swab on culture medium immediately after sampling
Rinsing of swab in 1 0 ml saline followed by plating of rinsing fluid immediately after sampling

abs for quantitative assessment *of wound* conta ation in elective surgery

very few bacteria were recovered from the unds there being a lower rate for *S aureus* as npared to *E coli* The time lapse between itamination and sampling influenced the recoy rate whereas the size *of the wound did not* ie manner in which swabs *were treated to release* mpled bacteria onto the culture medium was iportant

The findings will be discussed after a brief mment on the experimental model

The sides of a n g prot ded n c

periments Wounds were inoculated with a small olume (0 1 ml) of bacterial suspension A larger

ound rather than the wound tissue surface would ave been sampled

Although great care was taken to standardize all teps considerable variations in the results were ncountered Even within experiments coefficient f variation was high and occasionally reached 00% In two experiments (not included) aimed at stimating the variation ten swabs were used imultaneously to sample one wound Variation etween swabs was 42% and 65% for the two xperiments A further analysis showed that 95% onfidence limits of one and a half logarithms in ither *direction* might be involved in predicting wound bacterial population from wound culture count Variations to the same *extent* were appea rently experienced by *Scheibel et al* (1978) *in a* similar experimental setting

The low recovery of bacteria from the wound is probably dependent on several factors and should be viewed as secondary to dynamic processes taking place in the wound the net result of which is exemplified in Fig 1

A swab takes up *70% of a bacterial inoculum* from an agar surface (*Nystrom* 1978) but uptake is *probably considerably lower from a wound due to* bacteria being trapped in the rough wound surface

Volume distribution i e dilution of bacteria in the wound fluid and passive or active penetration of the wound *surface followed by* peripheral dissemi *nation of* bacteria in the tissues begins within the first hour (*Dhingra et al 1976*)

The biological mechanisms of the interaction between *bacteria and* live tissue are extremely *complex* both due to the bacteria and to the host *and these* vary for different bacteria and different *strains of* the same bacterial species (*Smith* 1976 *1977) This may* be exemplified by the differences in *recovery* of *S aureus* and *E coli* found in this *study and* by *Scheibel et al* (1978) with other *micro-organisms*

Adhesion of bacteria to animal cells is considered *to be a rapid* process (*Mardh & Westrom* 1976 *Bartelt & Duncan 1978*) and appears to be a *characteristic* of the bacteria in colonizing its specific biotope as well as a prerequisite for successful invasion *of host* by many bacterial pathogens (*Savage 1972 Gibbons & van Houte* 1975 *Eden et al* 1976) Adhesion probably takes place in a wound as *can be* inferred from studies on wound cleansing by high pressure irrigation (*Rodeheaver et al* 1975 *Hamer et al 1975 Brown et al* 1978) In addition to adhesion there *is also the* possibility of aggregation or agglutination of *bacteria in* the wound thus producing *microcolonies giving* fewer colony forming units

Specific immunological and non specific *humoral*

TABLE 3 *Influence of Wound Size on Quantitative Recovery of* Staphylococcus aureus *from Experimental Wo… Pigs by Swab Sampling*

Expt No	Inoculum per wound	No of wounds	Length of wound mean (mm)	Recovery per wound Mean ± SEM CFU	Time of samp… (min)	
1	1 0 × 10⁴	9	34	172 0 ± 33 3	10	N…
	1 0 × 10⁴	10	85	144 2 ± 17 5	10	
2	4 9 × 10⁴	7	42	683 9 ± 183 6	10	NS
	4 9 × 10⁴	10	83	718 5 ± 111 8	10	
3	5 1 × 10⁴	10	41	463 3 ± 107 6	30	NS
	5 1 × 10⁴	10	95	339 1 ± 52 9	30	

N S = not significant

Effect of Wound Size on Recovery

Using parallel experiments the influence of wound size was tested by comparing the standard wound of about 40 mm with a wound twice that length Wounds in sets of ten for each size were seeded with *S aureus* and sampled at 10 or 30 min

Recovery from the bigger wound did not differ significantly from that of the smaller wound (Table 3)

Effect of Consecutive Sampling

To determine the significance of the first culture compared with a second or third culture from the same wound, the following experiment was per… med one wound was seeded with 7 5 × 10⁴ C… and 10 min later the wound was sampled … consecutive times

A linear decline in recovery was observed … semilogarithmic system (Fig 3) Recovery … 0 4 % for the first culture and cumulative reco… after ten cultures was 0 7 %

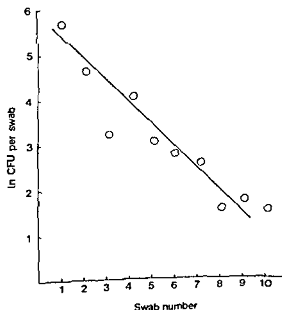


Fig 3 Recovery using ten consecutive swabs from one wound seeded with 5 × 10⁴ *Staphylococcus aureus* and sampled after 10 min Recovery was 0 4 % after the first swab and cumulative recovery *after ten* swabs was 0 7 %

Effect of Different Swab Handling

This part of the study was aimed at determin… the release of sampled bacteria from the swab u… three different swab handling procedures

Four sets of ten seeded wounds were samp… with three simultaneous swabs One swab … immersed in modified Stuart s transport med… (*Gastrin et al* 1968) kept at +4 °C and strea… onto the culture plate the following day in orde… mimic the procedure used in clinical practice … bacteriological specimens The second swab … streaked onto the culture plate immediately af… sampling The third swab was placed in a tube … 1 0 ml saline The tube was shaken on a »whi… *mixer« for one min after which the swab* … squeezed against the tube wall to remove as mu… fluid as possible and then discarded The t… contents were emptied onto the culture plate wh… was rocked until the fluid covered the agar surfa…

Immediate streaking gave a three times hig… yield and the rinsing procedure a four times hig… yield as compared to storage of the swab in Stua… transport medium (Table 4)

DISCUSSION

The investigation was undertaken to determine … efficacy of sampli…

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defence mechanisms as well as phagocytosis and intracellular killing of bacteria are probably operative within the first hour (*Hohn et al* 1977[b], *Hohn et al* 1977[a])

The low recovery of bacteria from the wound and the decline in recovery with time may then be viewed primarily as due to physical factors inherent in the sampling procedure as such and secondly due to adhesion of bacteria to the tissues volume distribution and humoral and cellular inactivation and destruction of bacteria

Experimental investigations in animals (*Edlich et al* 1968 *Kelly* 1978) and in man (*Elek & Conen* 1957) have demonstrated the high level of bacteria necessary to produce suppurative infection in undamaged tissue Bacterial concentrations below 10^5–10^6 CFU seem to be eliminated without clinical infection On the other hand quantification of the microflora in the wound in clean surgery (*Burke* 1963 *Lilly et al* 1970 *Sanderson & Bentley* 1976) and in potentially contaminated surgery (*Taylor* 1961 *Raahave* 1974 1976 *Nystrom* in preparation) has demonstrated bacterial populations at the hundred level or lower and sometimes cultures are negative while the wound is subsequently found to be infected

Though potentiating factors such as sutures or ischemia which are always present to some extent in operation wounds reduce the number of bacteria necessary to produce suppuration (*Elek & Conen* 1957 *Edlich et al* 1968) there is still a discrepancy between experimental and clinical experience Taking into account that the detection level in this study and in the work of *Scheibel et al* (1978) was in the range of 10^2 bacteria and higher as time elapsed after contamination this would appear to bring clinical results in accordance with experimental data

There is experimental and clinical evidence that post operative wound infection may occur subsequent to bacteriaemia due to remote infection (*Robson et al* 1973) This might explain some of the infections in patients with negative incisional wound cultures

The cotton swab is commonly used for sampling microbiological specimens in clinical practice Wound swabbing together with velvet pad imprint (*Raahave* 1974) and wound irrigation (*Taylor* 1961) are surface sampling methods as opposed to tissue biopsy homogenization (*Krizek & Robson* 1975) which is a volume sampling method Surface sampling would appear to be the method of choice for per operative use in elective surgery (*Raahave* 1974)

Present evidence suggests that the recovery rate of bacteria does not differ much by the various surface sampling methods but modifications which aim at increasing the uptake of bacteria from the wound or the collection of bacteria from the sampler are important in increasing the sensitivity of the methods (*Raahave* 1974 1975a 19[illegible] *Scheibel et al* 1978 *Nystrom* 1978)

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STUDIES ON FUNGAL FLORA IN HAIR FROM DOMESTIC AND LABORATORY ANIMALS SUSPECTED OF DERMATOPHYTOSIS

I Dermatophytes

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Aho R Studies on fungal flora in hair from domestic and laboratory animals suspected of dermatophytosis I Dermatophytes Acta path microbiol scand Sect B 88 79–83 1980

Hairsamples of domestic and laboratory animals suspected of dermatophytosis were examined for the presence of dermatophytes A nutritionally poor base medium developed by the author was successfully used in the isolation and identification of dermatophytes Casein medium supplemented with vitamins and Sabouraud liquid medium were used in special cases Dermatophytes were isolated in 36 of 331 samples (10 9 %) The dermatophytes recovered were *Microsporum canis* 13 isolates from cat 4 from dog 1 from horse *Trichophyton mentagrophytes var granulare* 3 isolates from dog 3 from horse 2 from guinea pig and 1 from rabbit *Trichophyton terrestre* 1 isolate from dog Eleven of the 13 feline isolates originated from house cats and the relative frequency was higher among the purebred cats Two of the cat isolates were connected with human dermatophytosis

Key words Dermatophytes fungal diagnostic medium fungal zoonosis

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Accepted as submitted 21 ix 79

Dermatophytosis a fungal infection of the keratinized layer of the skin hair and nails is caused by dermatophytes of the *Microsporum Trichophyton* and *Epidermophyton* families According to *Ajello* (2) 21 *Trichophyton* and 15 *Microsporum* species are known of which at least ... are pathogenic to man or animals or both *Epidermophyton floccosum* is pathogenic to humans but seldom reported to be pathogenic to animals (1)

A few reports have been published on animal dermatophytosis in Finland during the last three decades (16 22 23 24)

The present investigation was undertaken to elucidate the occurrence and importance of dermatophytosis in domestic and laboratory animals in Finland and forms part of a more comprehensive study on fungal flora of skin in suspected cases of dermatophytosis

MATERIAL AND METHODS

An appeal to veterinarians to send diagnostic material was published in the Journal of the Finnish Veterinary Association No 4 1977 The instructions were those suggested by *Muller & Kirk* (19) Veterinarians were [illegible]

[illegible] Department of Microbiology and Epizootology at the College of Veterinary Medicine Helsinki Of the 331 samples submitted (83 %) were sent by 27 veterinarians from greater Helsinki (249 from small animals 3 from laboratory animals 23 from large animals) The 56 samples (17 %) from other parts of Finland included 50 from southern Finland other than Helsinki 3 from south-eastern Finland and 3 from western Finland (28 from small and 28 from large animals)

The source of samples according to animals species is given in Table 1 Nail as well as hair samples were

STUDIES ON FUNGAL FLORA IN HAIR FROM DOMESTIC AND LABORATORY ANIMALS SUSPECTED OF DERMATOPHYTOSIS

I. *Dermatophytes*

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Aho, R. Studies on fungal flora in hair from domestic and laboratory animals suspected of dermatophytosis. I. Dermatophytes. Acta path. microbiol. scand. Sect. B, *88*: 79–83, 1980.

Hair samples of domestic and laboratory animals suspected of dermatophytosis were examined for the presence of dermatophytes. A nutritionally poor base medium developed by the author was successfully used in the isolation and identification of dermatophytes. Casein medium supplemented with vitamins and Sabouraud liquid medium were used in special cases. Dermatophytes were isolated in 36 of 331 samples (10.9%). The dermatophytes recovered were *Microsporum canis*: 13 isolates from cat, 4 from dog, 1 from horse; *Trichophyton mentagrophytes var. granulare*: 3 isolates from dog, 3 from horse, 2 from guinea pig and 1 from rabbit; *Trichophyton terrestre*: 1 isolate from dog. Eleven of the 13 feline isolates originated from house cats and the relative frequency was higher among the purebred cats. Two of the cat isolates were connected with human dermatophytosis.

Key words: Dermatophytes; fungal diagnostic medium; fungal zoonosis.

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Accepted as submitted 1.x.79.

Dermatophytosis is a fungal infection of the keratinized layer of the skin, hair and nails. It is caused by dermatophytes of the *Microsporum*, *Trichophyton* and *Epidermophyton* families. According to *Ajello* (2) 21 *Trichophyton* and 15 *Microsporum* species are known, of which at least [illegible] are pathogenic to man or animals or both. *Epidermophyton floccosum* is pathogenic to humans but seldom reported to be pathogenic to animals (1).

A few reports have been published on animal dermatophytosis in Finland during the last three decades (16, 22, 23, 24).

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MATERIAL AND METHODS

An appeal to veterinarians to send diagnostic material was published in the Journal of the Finnish Veterinary Association No. 4, 1977. The instructions were those suggested by *Muller & Kirk* (19). Veterinarians were asked to wipe the coat with water or 70% ethanol, store the samples in a refrigerator and send them once a week to the laboratory. Samples were collected by veterinarians between *April 1977* and May 1979 and sent to the Department of Microbiology and Epizootology at the College of Veterinary Medicine, Helsinki. Of the 331 samples submitted (83%) were sent by 27 veterinarians from greater Helsinki (249 from small animals, 3 from laboratory animals, 23 from large animals). The 56 samples (17%) from other parts of Finland included 50 from southern Finland other than Helsinki, 3 from south-eastern Finland and 3 from western Finland (28 *from small and 28 from large animals*).

The source of samples according to animal species is given in Table 1. Nail as well as hair samples were

TABLE 2 *Age Specific Distribution of Dermatophytes in Dog and Cat*

...al ...s	Dermatophytes isolated by age group																	
	<1 year			1 2 years			2–4 years			>4 years			age unknown			total		
	n	+	%	n	+	%	n	+	%	n	+	%	n	+	%	n	+	%
	17	2	11.8	42	3	7.1	45	1	2.2	98	2	2.0	4	0	0	206	8	3.9
	13	7	53.8	18	2	11.1	8	2	25	20	2	10	2	0	0	61	13	21.3

TABLE 3 *Sex Specific Distribution of Dermatophytes in Dog and Cat*

...nal ...es	Female			Male			Total			Sex unknown		
	n	+	%	n	+	%	n	+	%	n	+	%
	111	3	3.6	83	4	4.8	194	7	3.6	12	1	8.3
...payed/ ...astrated	2	0	0	3	0	0	5	0	0			
	25	4	16.0	32	8	25.0	57	12	21.1	4	1	25.0
...payed/ ...astrated	5	0	0	9	0	0	14	0	0			

...ubgroup included in the group above

TABLE 4 *Breed Specific Distribution of Dermatophytes in Cat*

	Occurrence of dermatophytes					
	Distribution by breed			In summary		
	n	+	%	n	+	%
...mestic short hair cats	39	5	12.8	39	5	12.8
...rebred cats				22	8	36.4
Persian	15	5	33.3			
Colorpoint persian	1	0	0			
Siamese	2	1	50			
Burmese	2	1	50			
Rex	1	1	100			
Abessinian	1	0	0			
...tal				61	13	21.3

...rew also on the base medium but quickly growing ...ontaminants soon interfered with the growth

Microscopic examination of the native preparates ...id not prove worthwhile because of the rarity of ...pores in specimens from cases of dermatophytosis

The positive cases originated in towns and ...illages of southern Finland except for one *M canis* ...solate from horse and three *T verrucosum* isolates ...rom cow which were sent from western Finland

Of 32 animals treated with antifungal drugs before the examination two cats and one dog tested positive for dermatophytes

In ten cases pets were examined mainly because of the pet owners dermatophytosis The skin infections of two pet owners (diagnosed by physicians of the Helsinki University Hospital) and their cats were culturally proved to be caused by *M canis* Six of the owners were demonstrated to suffer from

received from two dogs No differences in quality of samples of small animals could be seen with the naked eye but approximately one third of the samples of large animals were distinctly dusty This was verified by microscopic examination of native preparates

Of the 206 dogs examined 188 were purebreds 12 were mixed breed and 6 were of undesignated breed The most common breeds were German shepherd (13 dogs) Westhighland white terrier (13 dogs) cocker spaniel (12 dogs) and dachshund (9 dogs) The breed distribution of cats can be seen in Table 4 Age and sex distribution of cats and dogs examined is displayed Tables 2 and 3

According to information supplied with the specimens 12 cats 18 dogs and 2 guinea pigs had been treated with local or systemic antifungal drugs in the two months before the fungal examination In ten cases the pet owner was suspected of having dermatophytosis and the pet was examined mainly for this reason All these animals were reported to show at least slight symptoms Some of the animals examined apparently had other diseases seven dogs and three cats had symptoms of hormonal imbalance one dog diabetes mellitus one dog acanthosis nigricans and four dogs demodicosis

Native preparations of each sample were mixed with potassium (5 g) glycerine (25 ml) distilled water (20 ml) solution and applied to the object glass and examined by a phase contrast microscope About twenty hairs of each sample were cultivated on a base medium developed by the author (5 g glucose 5 g peptone 15 g agar 1000 ml distilled water 20 iu/ml penicillin 40 iu/ml streptomycin pH = 6 6) Nail samples were kept 10 minutes in 70% ethanol then cut into small pieces and mounted on the base medium Besides the base medium hair samples of cow were also mounted on Casein thiamine inositol agar (8) and Sabouraud liquid medium (25) and hair samples of horse on Casein niacin acid agar (8) The incubation temperature was + 22 °C except for the cow hair cultures which were incubated at both + 2 + 35 °C The laboratory was equipped with U Plates and liquid medium cultures were incubat 6–8 weeks if necessary and examined every f

RESULTS

The distribution of the material by animals and the dermatophyte isolates by species is ted in Table 1 Dermatophytes were hair samples (10 9%) The two nail s examined were negative The age and sex frequencies of feline and canine dermatophytos given in Table 2 and 3 The breed sp distribution of dermatophytosis in cats can be in Table 4 Dermatophytes of dogs were is from seven purebred dogs (twice in dachshu once in Keeshond German shepherd fox doberman and boxer) in one positive case t was not known Fifty nine of the 61 cats exa were house pets The two strays examined positive

The nutritionally poor base medium was su fully used in isolation and identification of M *T mentagrophytes* and *T terrestre* The grow these species was detected within 4–7 day typical spores and pigments were produced 5–14 days Contaminant fungi e g *Aspergil Penicillium* species grew slower than in nu nally rich Casein agar *T verrucosum* (8 is was most successfully isolated on Sabouraud medium and in one case only on Casein thia inositol medium *T verrucosum* as a slow g

TABLE 1 *Occurrence of Dermatophytes in Hair Samples by Species and Host*

	Number of animals examined	Microsporum canis	Trichophyton mentagrophytes	T verrucosum	T terrestre	Percen of pos
Dog	206	4	3	–	1	3 9
Cat	61	13	–	–	–	21 3
Horse	27	1	3	–		14 8
Cow	19	–	–	8	–	42 1
Guinea pig	10	–	2	–	–	20 0
Rabbit	1	–	1	–	–	100 0
Cage bird	2	–	–	–	–	–
Goat	1	–	–	–	–	–
Chincilla	1	–	–	–	–	–
Rat	1	–	–	–		–
Mink	1	–	–	–	–	–
Lesser panda (*Ailurus fulgens*)	1					
	331	18	9	8	1	10 9

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In four cases of *T verrucosum* isolation in cattle, an outbreak of dermatophytosis on the farm was clinically reported A *M canis* infection in horse was connected with a stable epidemic of dermatophytosis, apparently introduced by a five-year-old stallion and spreading to two foals

DISCUSSION

The nutritionally poor base-medium was developed by the author with the idea that such a medium would favour the development of the fruit bodies which are valuable to the identification of the genera (7) From this point of view the medium proved to be successful (unpublished preliminary results) To what extent the lack of nutritients in the medium influence the spectrum and proportion of dermatophytes and contaminants cannot be more exactly estimated before comparative studies have been carried out However, dermatophytes which need vitamin addition for quick growth, e g *T verrucosum* presumably are unable to compete successfully with quickly growing fungi The addition of cycloheximide to the base-medium might also be of value in preventing the overgrowth of contaminants This will be studied in the future

Whether and to what extent the method applied, especially the collection and handling of samples led to false negative findings is one of the most important considerations as regards the reliability of the results The great amount of contaminant fungi in some of the specimens, especially those from large animals must have had a preventive influence upon the growth of dermatophytes Fast growing fungi which additionally may possess antagonistic properties greatly inhibit the growth of slowly growing species The time between obtaining the sample and cultivating it was less than two weeks which probably had no influence on the viability of dermatophytes (12 19) But many contaminants could have grown during the mailing period suppressing the growth of the dermatophytes

The collection of samples from animals with only slight symptoms would have been better carried out by the Mackenzie hairbrush-technique (3)

Antifungal treatment of animals before examination naturally may have influenced the results in respective cases However *Wright* (26) has presented evidence that even after 8 weeks treatment with griseofulvin, isolation of dermatophytes may still be possible

According to *Muller & Kirk* (19) young most frequently suffer from dermatophy our material only 8 3% of dog hair sam 21 3% of cat hair samples came from anim than one-year old, but the highest i dermatophytosis was nevertheless found t sex disposition could be statistically pr dermatophytosis of dog and cat, but derma sis was found in 36 4% of purebred cats compared with 12 8% of domestic short examined (X^2-test $p<0\ 05$) This may de the contacts with other cats in cat shows mating, etc *Kushida* (14) has noticed t breed disposition No significant breed dis in relative frequency of positive dog isol found

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EXPERIENCE WITH AN INDIRECT (PASSIVE) HAEMAGGLUTINATION TEST FOR THE DEMONSTRATION OF RUBELLA VIRUS ANTIBODY

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G Haukenes Experience with an indirect (passive) haemagglutination test for the demonstration of ubella virus antibody Acta path microbiol scand Sect B 88 85-87 1980

A new indirect (passive) haemagglutination technique (Rubacell Abbott) has been evaluated on the basis of a material of 1 792 sera sent for serological diagnosis of a rubella infection or determination of immunity status The test procedure is very simple IHA antibodies appeared about two weeks later than HI antibodies and seemed to persist for life Two fields of application for the IHA technique are discussed 1 As a supplement to other tests because of the late appearance of the antibodies 2 In screening for immunity status The IHA combines ease of performance with high sensitivity

Key words Rubella indirect haemagglutination passive haemagglutination

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ı rubella infection is usually diagnosed serologi y by demonstration of seroconversion or a rise ıtre by the haemagglutination inhibition (HI) the haemolysis in gel (HIG) test or one of the s using labelled anti immunoglobulin The re ıtive antibodies can be detected a few days after appearance of the rash In most cases the titre ches a maximum level within one week. newhat with the complement fixation test If the ı serum specimen is taken in the second week of disease or later a rise in titre may often not be nonstrable In such cases the diagnosis can be ıde only by demonstrating specific IgM

An indirect (passive) haemagglutination (IHA) t has been developed by Abbott Laboratories The tibodies detected by this IHA persist after a bella infection (4) *Meurman* (3) collected several rum specimens from 31 patients with acute bella and compared the IHA with other tests He ncluded that the IHA antibodies appeared two to ree weeks later than HI antibodies so that the ıding of a positive HI and a negative IHA was athognomonic of an acute rubella infection and ıade demonstration of IgM unnecessary

We have examined the IHA test on a material consisting of about 1 800 sera in order to evaluate its reliability and usefulness

MATERIALS AND METHODS

Sera

The sera were sent to the laboratory for diagnosis of rubella infection or determination of immunity status They were inactivated and examined either fresh on arrival or after storage at −20 °C

Serological Tests

The *standard HI test* was performed after absorption of the serum with kaolin at pH 9 and using sheep erythrocytes A *newly developed HI procedure* not requiring removal of non specific inhibitors (2) was run parallel with the standard test In brief sheep erythrocytes were sensitized by four haemagglutinating units of the haemagglutinin When the sensitized erythrocytes were mixed with an antiserum antibody inhibited the HA without interference by non specific inhibitors The *IHA* (Rubacell Abbott Labs) was performed as described by the manufacturer Twenty five µl serum diluted 1 13 5 was mixed with 25 µl stabilized human erythrocytes sensitized by a soluble rubella virus antigen using microtitre V plates The plates were taped shaken

for a few minutes and left at room temperature for 2 h or more before reading The HA patterns were distinctly negative or positive unless the sera had been taken during the first weeks after an acute rubella infection at the time of seroconversion Because of the small volume of the reactants (50 µl) only a limited number of sera should be set up before taping in order to avoid evaporation

Haemolysis in Gel (HIG)

This was performed on commercial plates (Orivir Orion Diagn Helsinki) A haemolysis zone of 5 mm or more was considered as a positive reaction (5)

Flotation Centrifugation

This was performed as described in (1) in cases where it was uncertain whether a very weak HI reaction was caused by antibody or inhibitor

RESULTS

A total of 1 792 sera were examined by three parallel methods standard HI new HI and IHA The IHA was performed qualitatively at dilution 1 13 5 unless a rise in titre in connection with a recent rubella was suspected The results obtained by the new HI procedure have been published elsewhere (2) They correlated almost completely with the results obtained by the standard HI test

All readings were made blind *i e* without knowledge of the history or the results of other tests When in doubt the IHA was repeated and often titrated When the test was repeated because of any disagreement only the first reading was recorded in order to evaluate the test as a single screening method for immunity status

When there was any disagreement between IHA and the HI tests an HIG test was performed and occasionally also floating centrifugation

When acute rubella infection was suspected the serum specimens were taken at the intervals recommended for the HI test If the second specimen was still IHA negative a third specimen was requested even when the diagnosis had been established The results are shown in Table 1 Fourteen sera were HI negative and IHA positive Six of these gave a false positive IHA reaction as defined by a negative HI and HIG test The six sera had been examined during a period when we had considerable difficulty in interpreting the IHA pattern They were re examined with a new batch of sensitized cells and were all IHA negative The other eight of the sera which showed positive IHA and negative HI showed a false negative HI reaction

The group negative IHA and positive HI comprised 131 sera 113 of which were from patients with an acute or recent rubella infection

TABLE 1 *Comparison of HI and IH*

Positive HI and IHA		1
Negative HI and IHA		
Negative HI and positive IHA		1
False positive IHA	6[a]	
False negative HI	8[b]	
Positive HI and negative IHA		1
Acute rubella	113	
False negative IHA	6[b]	
False positive HI	4[a]	
Unidentified	8[d]	
Total number of sera examined		1

[a] Negative HIG
[b] Positive HIG
[c] Seroconversion later
[d] Suspected rubella One serum specimen only

They all showed seroconversion later Eight ... listed as non identified They were from c... suspected rubella but the diagnosis wa... verified since we received only one serum ... men Six sera gave a false negative IHA re... When re-examined they were found to be ... positive Four sera gave a false positive HI re...

All sera were collected during a rubella ep... and pregnant women had usually contacte... doctor immediately when they had been ex... rubella or suspected a rubella infection Only ... sera were taken too late to obtain a rise in t... means of the HI test In our routine we exam... first serum sample for IgM when it is taken ... pregnant women with a history of rubella up ... months earlier Only in two cases cou... diagnosis be made by seroconversion in III ... stationary HI titres

We tried to calculate when the IHA ... positive in relation to the first day of the rash ... 106 patients with acute rubella we obtaine... information about the date of the rash The ... time from the first day of the rash to t... negative IHA test was 14 days (range 3 to 3... The average time for the first positive IHA ... days (range 5 to 68 days) The average negati... period was somewhere between 14 and 24 ...

When performed as a qualitative te... procedure is very simple and a trained te... can perform about 50 tests per hour

DISCUSSION

The new IHA method developed by ... Laboratories represents a new and val... the serological dia...

larly for the assessment of immunity status sults with a material of about 1 800 sera n the findings obtained by *Meurman* (3) from l material studying IHA in the diagnosis of rubella Laboratories which receive a great :r of specimens will possibly resort to one of :thods which can be automatized such as RIA LISA both when examining for immunity ise or specific IgM Other laboratories will to choose between HIG and one of the HI ques for the diagnosis of rubella infection

gical Diagn sis of a Rubella Infection

e IHA should never be used as the only test for ody when a rubella infection is suspected As a ement to the other tests which become positive days after the appearance of the rash the IHA give valuable information even at the time of of the first serum speciman A positive HI RIA or ELISA combined with negative IHA hly indicative of an acute rubella infection The osis must of course be confirmed by a rise in or by demonstration of rubella specific IgM a false negative IHA reaction may occur rever since acute rubella is suspected already n the first serum specimen is examined irmatory tests may be performed immediately unnecessary delay is avoided The advantage of as a supplementary test is thus obvious If a ratory does not have the capacity for examining antibody positive first serum specimens from nant women for IgM the IHA should be ormed in the absence of a rise in titre

rubella reinfection is suspected when a rise in is found without the presence of IgM and re there are no symptoms In two cases not uded in this material we suspected a reinfection these grounds The finding in both of them of a n and stable IHA titre at the time when the HI HIG titre was still rising in the absence of IgM e further support to our suspicion Later gnosis of a reinfection was verified for one of m when we obtained a serum sample that had n taken half a year earlier

If the titre obtained using the other tests has elled off the IHA may still show seroconversion a rise in titre Our findings agree with those tained by *Meurman* (3) as regards the average ne of se

reening for Immunity Status

The performance of the IHA test is very simple d the price is reasonable IHA antibodies seem to for life The number of false positive reactions was low This could be related to a special batch of erythrocytes and such reactions have not been recorded since It has been argued that the late appearance of IHA antibodies is a drawback when screening for immunity status Obviously very few individuals can be proved to be in the two to three weeks seronegative period after an asymptomatic rubella infection and the vaccine will certainly do them no harm Another question is whether a quantitative method might pick up individuals with very high serum titres thus suggesting a recent infection and leading to examination for specific IgM This is of interest only when women are examined in the first trimester of their pregnancy It is difficult to predict the results of such a practice We have titrated rubella HI antibody in sera taken both early and late in pregnancy from about 2 000 women (unpublished) Three cases of seroconver sion and one reinfection were found Nineteen sera with HI titres of 640 or higher were examined fur ther by HIG and IHA and for specific IgM No signs of recent infection were detected It must be emphasized however that the sera were collected during an inter-epidemic period just before the start of an epidemic It remains to be seen whether the IHA may prove useful in this context The HA pattern may be very weak or doubtful for a con siderable period at the time of seroconversion

In our opinion screening for rubella immunity by means of this IHA should be performed only in laboratories that carry out serological diagnosis of rubella infection where IHA is included Only in this way can the reagents for the IHA test be check ed regularly for sensitivity and reliability If IHA is used to supplement another method of screening for immunity status recent infections may also be revealed Such screening will be carried out in our laboratory to evaluate further the place of the IHA in this context

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⊃ELLULAR FATTY ACID COMPOSITION OF *HAEMOPHILUS* SPECIES, *PASTEURELLA MULTOCIDA*, *ACTINOBACILLUS ACTINOMYCETEMCOMITANS* AND *HAEMOPHILUS VAGINALIS (CORYNEBACTERIUM VAGINALE)*

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Jantzen E Berdal B P & Omland T Cellular fatty acid composition of *Haemophilus* species *Pasteurella multocida Actinobacillus actinomycetemcomitans* and *Haemophilus vaginalis (Coryne bacterium vaginale)* Acta path microbiol scand Sect B 88 89–93 1980

The fatty acid composition of 35 *Haemophilus influenzae* strains was found to be grossly similar and characterized by relatively large amounts of 14 0 3-OH 14 0 16 1 and 16 0 The three C_{18} fatty acids 18 2 18 1 and 18 0 were also present but in much lower concentrations This general pattern was also found for most of the other species of *Haemophilus* examined (*H aegyptius H aphrophilus H canis H gallinarum H haemolyticus* and *H parainfluenzae*) Small but distinct quantitative discrepancies were detected for *H ducreyi* and the haemin independent species *H paraphrohaemolyticus H paraphrophilus* and *H suis Actinobacillus actinomycetemcomitans* was found to be indistinguishable from *H influenzae Pasteurella multocida* also exhibited a fatty acid pattern closely related to that of *Haemophilus* but could be distinguished by its higher concentration levels of the C_{18} fatty acids The fatty acid pattern of *H vaginalis* was considerably different from those of the other species examined This species lacked 3 OH 14 0 and 18 2 and contained small amounts of 14 0 and 16 0 whereas 18 1 and 18 0 were the major constituents

Key words Bacteria fatty acid composition gas chromatography taxonomy

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Numerous taxonomic studies based on bacterial …ty acid composition have revealed clearly the …efulness of quantitative data on lipid constituents …taxonomic evaluations (1 13 14 17) Fatty acid …ofiles provide simultaneously several taxonomic …aracters and it has been shown that grouping …sed on fatty acid composition matches reasonably …ell the classification achieved by genetic parame…rs (5 7) However before this chemical technique …n be fully utilized in taxonomy many groups of …ell characterized bacteria and if possible several …rains of each species must be analysed preferably …y a standardized procedure

We are considering in particular the use of fatty …cid profiles in the identification of unusual clinical isolates which are difficult to assign to genus or species and have …

…cation deals with the fatty acid composition of several *Haemophilus* species and of *Pasteurella multocida* Two strains of *Actinobacillus actinomy cetemcomitans* and one strain of *H vaginalis (Corynebacterium vaginale)* were also included in the study since their relatedness to *Haemophilus* appears to be unclear at present The fatty acid composition of *Francisella tularensis* has been published elsewhere (8) and studies of *Bordetella* and *Brucella* are in progress

MATERIALS AND METHODS

Bacterial Strains, Growth and Harvesting

Thirty-five *Haemophilus influenzae* strains were analysed Fourteen were obtained from the National Collection of Type Cultures, London, designated by the following NCTC numbers 4842, 7279, 7918, 8143, 8455, 8465, 8466, 8467, 8468, 8469, 8470, 8472, 8473, 10479 Six serological reference strains (*T Omland*), *A51*, *B51*, *C51*, *D51*, *E51* and *F51* were received originally (1957) from *H C Engbak*, Statens Seruminstitut, Copenhagen, and two strains (E-Montenegro and f-Dingles) from *G Leidy*, Columbia University, N Y The remaining thirteen strains were own isolates

In addition, the following *Haemophilus* species were examined *H aegyptius* NCTC 8134, NCTC 8502, *H aphrophilus* NCTC 5886, NCTC 5906, *H canis* NCTC 1659, NCTC 10619, *H gallinarum* NCTC 3438, *H haemolyticus* NCTC 8479, NCTC 10659 *H parainfluenzae*, *NCTC 7857 NCTC 7857*, *NCTC* 10665 *H paraphrohaemolyticus* NCTC 10670 NCTC 10671, *H paraphrophilus* NCTC 10556, NCTC 10557, *H suis*, NCTC 4557, NCTC 6359, *H ducreyi* ATCC 27722, and one strain received from *K Odegaard* National Institute of Public Health, Oslo

The two *Actinobacillus actinomycetemcomitans* strains NCTC 9709, NCTC 9710 and the single representative of *H vaginalis* NCTC 10287 were obtained from the National Collection of Type Cultures

Of the sixteen *Pasteurella multocida* strains examined fifteen were received from *S D Henriksen* University of Oslo These were serological reference strains received originally from Japan Kobe 5 (1 A) M4 (1 A) Kobe 6 (2 0) P 27 (2 0) P 8 (3 A) M 17 (4 0) TS 8 (5 0) VA 3 (5 A) R 4 73 (6 B) Bunia (6 E) PM (7 A) 147 (8 ?) Liver (9 A) and 989 (11 B) One strain NCTC 3195 was obtained from the National Collection of Type Cultures

The strains were maintained and cultivated on chocolate agar (10% heated defibrinated horse blood in blood agar base (Difco)) One single batch of medium was used for cultivation Incubation was performed for 24 hours at 37 °C in the presence of 5% CO_2 The bacteria were harvested in phosphate buffer (0 5 M pH 7 2) centrifuged and washed twice by distilled water before freeze drying

Chemicals and Chemical Analyses

Solvents of *pro analysis* grade were distilled before use Standards of fatty acid methyl esters were obtained from Applied Science Laboratories Inc , State College Penn , USA

Dried bacterial cells (1–10 mg) were methanolyzed by 2 N HCl in dry methanol as described previously (6 7) Fatty acid methyl esters were analysed on a Hewlett Packard 5710 gas chromatograph equipped with a flame ionization detector Two standard 2 m glass columns packed with either 10% SE-30 or CP-10 on Gas Chrom Q 100–120 mesh (Applied Science Laboratories Inc) were used routinely Additional analyses were perfor med on a 25 m SCOT SE-30 Column (C Middelburg, The Netherlands)

Peak identifications were obtained by c retention data with those of authentic standards o columns Hydroxy fatty acids were i *trifluoroacetylation and rechromatography* (7) tural elucidations were verified by gas chro mass spectrometry on a Hewlett Packard chromatograph mass spectrometer instrument with a 2 m long 1% OV-1 glass column

RESULTS

All thirty-five *H influenzae* strains grossly similar fatty acid patterns w *relatively large amounts of* 14 0 (see Ta nomenclature), 3-OH-14 0, 16 1 and 1 three C_{18} fatty acids, 18 2, 18 1 and 18 0 generally present but in much lower (Table 1) All recognized serotypes (a to Pittman scheme) and also the biotypes *Kilian* (11) were represented However n tion between fatty acid composition and s or biochemical characteristics could be

The fatty acid pattern found for *H* *was shared by the other haemin requir* except for *H ducreyi* which differed slig higher concentration of 14 0 and a lower tion of 16 0 Species of the haemin in group constituted a more heterogeneous *aphrophilus*, *H gallinarum* and *H para* were almost indistinguishable from the *requiring species whereas H paraphrol cus H paraphrophilus* and *H suis* show discrepancies in the 14 0 and 16 0 concent comparison to the *H influenzae* pattern

A actinomycetemcomitans could not guished from *H influenzae* or *H aphrop* *P multocida* exhibited a fatty acid pattern *that of Haemophilus* (Table 1) How indicated in Fig 1, this species could ge distinguished by a higher level of the C_{18} f On the other hand, as could be exp *vaginalis* exhibited a very different pattern It lacked 3 OH 14 0 and 18 2 and conta *small amounts of 14 0 and 16 1, wher* 18 1, and 18 0 were the most abundant ents

DISCUSSION

In an extensive taxonomic study of genus *philus* (11), Kilian recently showed the gu cytosine (G + C) content of DNA to be in t *of 37 to 44 mole per cent All haemin-i* species exhibited a G + C content below cent, whereas the haemin-independent

I *Percentage Cellular Fatty Acid Composition of* Haemophilus *Species* Actinobacillus actinomycetemcomitans Haemophilus vaginalis (Corynebacterium vaginale) *and* Pasteurella multocida[1]

	%G + C of DNA[3]	ALA → porphyrins[2]	V factor requirements[2]	Fatty Acid						
				14 0[4]	3-OH 14 0	16 1	16 0	18 2	18 1	18 0
ae ins)	38 7	–	+	12 7[5] 8 6–16 9[6] 2 30[7]	11 6 9 9–13 4 1 08	31 4 24 3–35 5 2 41	40 6 36 4–46 5 2 41	0 2 0 0–0 5 0 15	0 5 0 2–0 8 0 16	2 4 1 1–5 6 1 13
us	38 7	–	+	16 2 13 8	12 8 14 8	28 0 24 5	39 9 40 5	0 2 0 4	0 5 0 4	2 4 4 4
h lus	42 0	+	–	15 8 23 0	14 7 13 8	26 3 22 7	38 7 38 0	0 4 0 3	0 4 0 4	2 7 1 3
	38 1	–	–	17 4 13 5	13 3 13 8	24 7 24 7	40 1 40 6	0 2 0 7	1 1 0 8	2 9 4 9
rum	42 2	+	+	10 7	12 4	29 1	41 3	0 5	0 7	5 6
lyt cus	38 5	–	+	23 2 12 4	17 7 23 8	26 5 33 4	28 9 40 3	0 4 1 0	0 9 2 1	1 4 9 3
fluen ae	39 7	+	+	13 3 12 7	13 8 16 2	32 0 33 8	36 7 33 1	0 6 0 7	1 0 1 1	2 1 2 0
hro *cus*	40 6	+	+	34 3 43 0	17 6 17 3	21 9 19 6	16 8 18 2	0 2 0 4	0 5 0 5	0 5 0 6
hroph lus	41 9	+	+	22 0 21 7	14 4 15 0	26 6 26 7	33 9 34 6	0 6 0 3	1 0 0 8	0 9 0 7
	41 5	+	+	8 6 6 7	12 8 11 1	33 0 42 7	35 4 26 1	1 0 0 6	1 2 6 5	6 7 5 7
s	37 8	–	–	24 8 25 4	10 2 10 8	35 3 34 1	26 3 24 7	0 3 0 4	1 0 0 9	0 8 0 9
omycetem	42 7	+		12 2 18 7	18 1 15 0	26 9 24 6	37 3 38 4	0 3 0 3	0 7 0 9	3 1 2 1
al s	42 ± 1[8]			4 4	–	2 0	54 8	–	27 3	10 4
c da ra ns)	36 5–40 1[9]	+	–	10 7[5] 8 0–12 8[6] 1 31[7]	12 9 12 2 14 7 0 77	29 3 23 0–32 0 2 07	38 7 36 ?–42 0 1 43	1 9 1 0–3 4 0 61	1 7 0 7–3 0 0 50	4 6 3 5–6 4 0 83

See text for experimental details
See text for further description
Data of *Kilian* (11)
Key to the fatty acid designation the figure in the front of the colon indicates the number of carbon atoms in the chain the figure after the colon, the number of double bonds the symbol 3-OH indicates a hydroxy group and its position
Mean value
Range among strains
Standard deviation
Data of *Lapage* (12)
Data of *Belozersky & Spirin* (2)

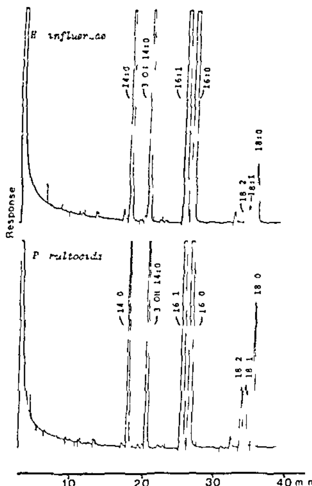


Fig 1 Typical gas chromatographic fatty acid methyl ester profiles of a *Haemophilus influenzae* and a *Pasteurella multocida* strain

The bacterial cells were digested by 2 N HCl in methanol extracted and trifluoroacetylated before injection on the 25 m SCOT SE 30 column

G + C values above that level These two main groups of *Haemophilus* could not be revealed clearly by the fatty acid patterns Thus all haemin requiring species except *H ducreyi* and the two haemin independent species *H aphrophilus* and *H gallinarum* shared the *H influenzae* pattern whereas the remaining four haemin independent species showed small but significant deviations both from each other and from *H influenzae* (Table 1)

Previous results have shown that only species of a certain phylogenetic distance can be distinguished by their fatty acid composition Within *Moraxella* most species could be differentiated (7) On the other hand within the *Enterobacteriaceae* where speciation is based mainly on serological characteristics only a few groups could be differentiated (9) The taxonomic distances between the *Haemophilus* species are apparently too small to be clearly reflected by their fatty acid compositions

In the latest edition of Bergey s Manual (4) the species *A actinomycetemcomitans* is excluded the genus *Actinobacillus* and listed as species in*c*. *sedis* (16) *Kilian* (11) demonstrated a close r of this species to *H aphrophilus* and discusse possibility of its inclusion in genus *Haem* We were not able to distinguish *A actinom comitans* from *Haemophilus* by our method T the fatty acid data support a taxonomic revisi this direction

The taxonomic position of *H vaginalis* is L questionable As pointed out by *Lapage* (1 Bergey s Manual this species does not be genus *Haemophilus* Its association with the (*nebacterium* genus as suggested by *Zinn Turner* (19), is also unlikely since its cell wa diaminopimelic acid and contains 6-deo instead of arabinose *Lapage* accordingly r mends retaining the name *H vaginalis* un appropriate taxonomic position for this orga revealed In terms of fatty acid compositi species differed from most Gram negative ba by the absence of hydroxy fatty acids (13) The acid pattern of *H vaginalis* was also different that reported for *C diphtheriae* and *C acnes* well as from a group of pleomorphic soil b *(C fascians C michiganese C sedipomicum insidosum)* (15) These two groups were characterized by branched fatty acids as constituents Further comparative studies on composition may assign »*H vaginalis*« to a d genus

Haemophilus Pasteurella and *Actinob* share a number of phenotypic characters and t G + C contents are in the same range (10 11) numerical taxonomic study including represe ves of these three genera *Sneath & Johnson* obtained four clusters of relatively high co ph correlation coefficient On this basis the auth suggested that these groups should form the nucl of a new family Evidently the similarity in f acid composition of the species in this st supports this idea and additional compara studies of lipid composition might be of some va for evaluation of the taxonomic distances am these bacterial groups

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BINDING OF ENTEROTOXIN FROM *CLOSTRIDIUM PERFRINGENS* TYPE A TO LIVER CELLS *IN VIVO* AND *IN VITRO*

The Enterotoxin Causes Membrane Leakage

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Skjelkvåle R Tolleshaug H & Jarmund T Binding of enterotoxin from *Clostridium perfringens* type A to liver cells *in vivo* and *in vitro* The enterotoxin causes membrane leakage Acta path microbiol scand Sect. B *88* 95–102 1980

Enterotoxin from *Clostridium perfringens* was shown to retain its biological activity after labelling with 125I When injected intravenously into mice and rats most of the radioactivity in the organs was present in the form of intact toxin Studies of the tissue distribution of labelled enterotoxin showed the largest amounts in the liver where the activity reached a maximum 10–15 min after administration The highest concentration per g tissue was found in liver and kidneys The radioactivity was excreted in the urine as a mixture of intact labelled toxin and low molecular weight degradation products *In vitro* studies with purified parenchymal liver cells showed rapid release of lactate dehydrogenase (LDH) during treatment with enterotoxin thus indicating severe membrane damage

Key words *Clostridium perfringens* enterotoxin membrane leakage

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Clostridium perfringens produces a variety of biologically active metabolites among which is an enterotoxin associated with outbreaks of food poisoning (23)

In cases of food poisoning the enterotoxin acts mainly on the intestinal tract and causes diarrhoea So far little is known about the mechanism by which the enterotoxin causes membrane transport alterations metabolic disturbances and tissue damage leading to clinical manifestations

The disturbances in electrolyte and fluid transport due to the action of the enterotoxin have been studied in experimental animals (11 13) Epithelial desquamation has been induced in ileal loops treated with whole cells (4) or highly purified enterotoxin (12 14) The lethal nature of the enterotoxin has been established after intravenous or intraperitoneal injection resulting in death of experimental animals with hyperaemic small intestinal mucosa and congestion in liver lungs spleen and kidneys (16) No information is available about the distribution and binding of the toxin in the tissues After episodes of food poisoning a relatively high level of enterotoxin has been found in faeces and a significant increase in anti-enterotoxin titre in human serum has been demonstrated (21) Similar data have been obtained from human volunteers after ingestion of purified enterotoxin (22) It is not known whether this is the result of a local immune response or a general immune response due to absorption of the enterotoxin

The present report describes the plasma clearance distribution and excretion of purified 125I labelled enterotoxin after intravenous injection into mice and rats Experiments showing the effect of the toxin on the membranes of isolated rat hepatocytes are also included

MATERIALS AND METHODS

Experimental Animals

The majority of the experiments were performed on white male mice (NMRI/BOM) weighing 22–25 g while white male rats (MØLL/WISTAR) weighing 130–150 g were used in some experiments. All experimental animals were received from The Animal Department National Institute of Public Health Oslo

Enterotoxin

Clostridium perfringens type A strain NCTC 8239 was used for the production of enterotoxin in Duncan and Strong (DS) sporulation medium (3). The enterotoxin was purified by a modification (5) of the method described by *Sakaguchi et al* (19)

Iodination of Enterotoxin

Purified enterotoxin was labelled with ^{125}I using a modification of the chloramine T method (6) as described elsewhere (20). The following reagents were added to a 3 ml glass tube during continuous mixing (1) 10 µl carrier free Na ^{125}I (500 µCi) in 0.1 M NaOH (2) 10 µl 0.5 M phosphate buffer pH 7.0 (3) 10 µl toxin (1 mg/ml) (4) 10 µl chloramine T (1.2 mg/ml). The reaction was terminated after 40 s by the addition of 25 µg sodium metabisulphite dissolved in 300 µl 0.1 M phosphate buffer pH 7.0. The iodination was performed at room temperature. The labelled protein fraction was separated from the unreacted iodide by gel filtration of the reaction mixture through a Sephadex G 50 column (0.9 × 15 cm) equilibrated in phosphate buffered saline (PBS) pH 7.5 (0.1 M NaCl 0.05 M phosphate) containing 0.2% bovine serum albumin (BSA) and 0.02% sodium azide. Eluted fractions of labelled protein were combined and incorporation of ^{125}I was calculated. This procedure yielded a labelled preparation containing approximately 0.5 atoms of ^{125}I per molecule of enterotoxin. When tested in the guinea pig skin test (25) the labelled enterotoxin was found to be biologically active. An identical quantity of BSA was labelled by the same procedure except that the concentration of chloramine T was doubled. Labelled BSA was prepared for use in control studies. Labelled preparations were stored at −20 °C

Vascular Clearance of Enterotoxin and Localization of Enterotoxin in Tissues

^{125}I labelled enterotoxin was injected intravenously into one of the tail veins. In order to follow the clearance of toxin from blood and the distribution of toxin in tissues the animals were decapitated after ether anaesthesia at different intervals following injection

For determination of total radioactivity the organs were removed weighed and homogenized in 0.15 M KOH and the total radioactivity was measured

In preliminary experiments in which attempts were made to identify the labelled substances in the tissues the various organs were homogenized in PBS. One ml of the homogenate was precipitated with 2 ml 30% trichloroacetic acid (TCA) and left at 20 °C for 1 h. The suspension was centrifuged the pellet washed with 5% TCA and the radioactivity in the pellet and supernatants measured

Other samples of the homogenate were centrif... 5 min at 3 000 × *g* to remove the ... supernatant was subjected to sodium dodecyl s... (SDS)-acrylamide gel electrophoresis in glass ... × 6.5 cm) as described below. After electropho... gels were sliced and the radioactivity in each p... measured

Stability of ^{125}I labelled Enterotoxin in Blood

In vitro stability of labelled enterotoxin in b... studied by adding ^{125}I labelled toxin to heparin... blood (200 ng toxin/ml). The mixture was incu... 37 °C for 10, 30 and 60 min. Following incub... whole blood was centrifuged to produce pl... pellets were washed three times with PBS ... radioactivity in supernatants and pellets was m...

One ml samples of the plasma were treated ... equal volume of 30% TCA. The supern... separated and counted and the precipitate wash... times with 5% TCA. Determination was ma... radioactivity of each wash as well as that ... precipitate

Disc Gel Electrophoresis

Disc gel electrophoresis was performed acco... *Davis* (2) on 7% acrylamide gels with tris (hyd... thyl)-aminomethane glycine pH 8.5 as the ... buffer. Sodium dodecyl sulphate (SDS)-acrylar... electrophoresis was performed as described by ... *Osborn* (27). After electrophoresis the gels we... and the radioactivity in each piece was measure...

Binding Properties

The antibody binding properties of ^{125}I ... enterotoxin were tested in a solid phase syster... whole antiserum was used to coat polystyrene ... test tubes (8). Conditions for incubation dil... antiserum and buffers were as described previ...

Preparation of Purified Hepatocytes

Rat liver cells were prepared by a modificat... 26) of the collagenase perfusion method of ... *Friend* (1). Parenchymal and non parenchymal ... were separated by differential centrifugation (1... 95–99% of the hepatocytes excluded trypan b... purified hepatocytes were suspended in a ... incubation medium containing essential salts ... defatted BSA (26)

In vitro *Test for Membrane Damage*

Different concentrations of enterotoxin were ... suspensions of hepatocytes in the minimal mediu... 10^6 cells/ml) and incubated in a shaking water ... 37 °C. Portions of the cell suspensions were rem... different intervals and the hepatocytes were s... from the medium by centrifugation through ... phthalate (d = 1.05) as described previously (1... tested for permeability to trypan blue. The supe... was tested for lactate dehydrogenase activity ... below and LDH activity ... used as a... membrane damag...

mical Determ nations
ctate dehydrogenase (LDH) (L lactate NAD oxido-
tase E C 1 1 1 27) was assayed in a reaction

otein was determined according to the method of
D et al (10) with BSA as standard

ti ng Equ pment
adioactivity measurements were made in an LKB
lace 1280 automatic gamma counter

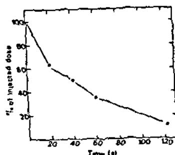


Fig 1 Clearance curve of [125]I labelled enterotoxin from *C perfringens* (37 μCi/μg) after intravenous injection into mice (100 ng per mouse) Each point represents the average of four measurements

RESULTS

LD50 for purified enterotoxin in mice is
oximately 2 μg (19 24) After intravenous
inistration of enterotoxin in the relevant dose
ge minute amounts of toxin are present in the
ues and these can be determined only by the use
abelled preparations

bility of [125]I labelled Enterotoxin in Blood
[125]I labelled enterotoxin was incubated with
arinized whole blood for different times and the
bility and binding to blood cells were calculated
ble 1) There was no increase in non TCA
cipitable radioactivity (later referred to as *free*
) *in vitro* during the incubation period Some 6–
is bound to the blood cells and 92% of the
ed [125]I labelled enterotoxin was found as TCA
cipitable material.

n (Fig 1) When blood samples were taken 20 s
er injection of toxin into a tail vein approxima
65% of the total injected dose could be
counted for in the whole blood if one assumes the
ood volume of the mice to be 7% of the body
weight After 120 s the amount of labelled toxin in the blood was reduced to approximately 10% of the injected dose Results from the study of radioactivity in tissues obtained at various times after challenge indicate that large amounts of the labelled enterotoxin were already taken up by liver and kidneys

Although the blood accounts for a minor part of the total radioactivity because of the rapid removal of toxin from the circulation interesting changes take place in blood radioactivity during the test period Ten m
toxin
table
were reduced to 78% and 66% respectively

Organ Distribution of [125]I labelled Enterotoxin

In order to ascertain whether the total radioactivity present in the tissues could be used as a measure of the presence of the toxin it was necessary to determine to what extent the radioactivity in the organs was present as TCA precipitable material presumably as intact toxin

Groups of 2–3 m

TABLE 1 In Vitro *Stability of [125]I labelled Enterotox n fr m* C perfringens *in Hepar ni ed Rat Blood at 37 °C*

Incubation time[a]	Free [125]I[b]	TCA precipitable radioactivity[c]	Radioactivity bound to blood cells
10 min	2%	92%	6%
30 min	1%	92%	6%
60 min	1%	92%	7%

[a] [125]I labelled enterotoxin (37 μCi/μg) was incubated at 37 °C with whole blood (200 ng toxin/ml blood)
[b] Non precipitable radioactivity referred to as «free [125]I»
[c] Trichloroacetic acid precipitable material

TABLE 2 *Radioactivity in Tissues after Intravenous Injection of* ^{125}*I labelled Enterotoxin from* C perfringens

Organ	Time after injection a) (min)	% radioactivity in precipitate b)	% radioactivity as intact toxin
Liver	10	98	97
	30	93	94
	60	91	
Kidneys	10	91	87
	30	89	87
	60	90	
Lungs	10	87	71
	30	77	60
	60	69	
Spleen	10	79	
	30	79	
	60		
Heart	10	70	
	30	79	
	60		
Intestines	10	91	87
	30	85	83
	60	83	

a) The animals were injected with 400 ng of ^{125}I labelled enterotoxin (37 μCi/μg)
b) Trichloroacetic acid precipitable material
c) As assayed by SDS gel electrophoresis
No observation made

the tissues were processed as described in Materials and Methods The major part of the radioactivity in the tissues tested was present in TCA precipitable form (Table 2) Analysis by SDS polyacrylamide gel electrophoresis showed that the radioactive material moved in the gel at the same rate as intact toxin From these data it would seem justified to use the distribution of the total radioactivity in the tissues as a measure of intact ^{125}I labelled toxin

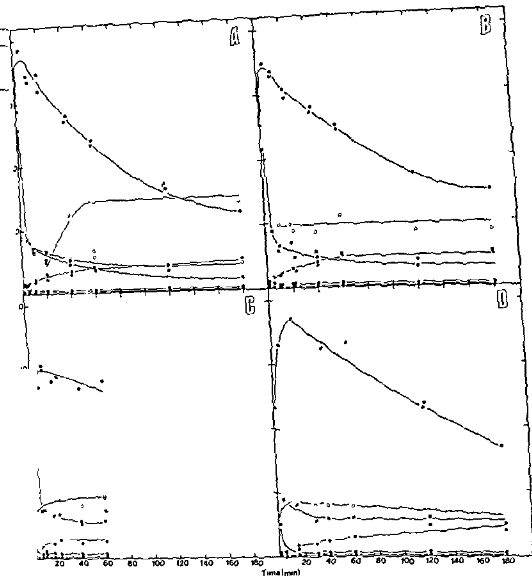
The results of typical experiments showing the distribution of radioactivity in the tissues after intravenous administration of labelled enterotoxin to mice and rats are given in Fig 2 Groups of animals were injected each group with different amounts of toxin and the data were expressed as percentages of the injected dose of radioactivity recovered per organ

It can be seen (Fig 2 A) that after intravenous injection of 100 ng ^{125}I labelled enterotoxin into mice, the largest amount of radioactivity was found in the liver where it increased rapidley to reach a maximum about 10–15 min after administration and then declined slowly

The uptake of ^{125}I labelled enterotoxin by the kidneys showed a similar pattern Lower le radioactivity were found also in lungs h intestines (including contents) spleen and skin radioactivity was low and is not shown in Fig

The results illustrated in Fig 2 A were obt after injection of very small amounts of toxin ng/mouse) Results from experiments where mice were injected with much higher doses of (100 ng ^{125}I labelled + 1 000 ng unlabe showed that the distribution in tissues and el tion from the organs were essentially uncha (Fig 2 B) When the injected dose of toxin increased to the LD_{50} value (100 ng ^{125}I labelled 2 000 ng unlabelled) the tissue distribution similar (Fig 2 C) but the elimination from the was dramatically reduced This is probably du reduced circulation The mice developed symp shortly after injection and none survived more 60 min It may be concluded from these results the tissue distribution and clearance rate from bl of intravenously administered enterotoxin were affected by the injected dose

The tissue distribution in rats is almost iden with that descri mice (F



2 Distribution of ^{125}I labelled *enterotoxin* from *C perfringens* (37 μCi/μg) in various organs after intravenous
tion into mice (A B and C) and rats (D)
roups of animals *were injected* with identical amounts of enterotoxin and killed and bled at different periods of
The organs were removed and processed as described in Materials and Methods The data are plotted as
ntage of the total injected radioactivity recovered per organ. Each point represents one animal.
100 ng ^{125}I labelled enterotoxin per mouse
100 ng ^{125}I labelled *enterotoxin* + 1 000 ng unlabelled enterotoxin per mouse
100 ng ^{125}I labelled enterotoxin + 2 000 ng unlabelled enterotoxin per mouse
800 ng ^{125}I labelled enterotoxin per rat
liver ■ blood ● lungs □ spleen
kidneys ☆ intestines ▲ heart ◬ urine

When the data in Fig 2 A were expressed as rcentages of the injected dose of radioactivity und per g of tissue (Fig 3), the labelled nterotoxin shows a preference for the liver and dneys with the highest affinity *to liver tissue*

After intraperitoneal injection of labelled enterotoxin the uptake in the organs occurred more slowly, and in most *cases the* radioactivity was lover than after intravenous injection (data not shown)

For comparison a group of mice was injected intravenously with 100 *ng* ^{125}I *labelled BSA* (speci-

activities demonstrated are primarily due to membrane damage but the rapid release of LDH from hepatocytes during treatment with enterotoxin would support this hypothesis

Recently *McDonel & McClane* (15) demonstrated that Vero (African green monkey kidney) cells had binding sites for *C perfringens* enterotoxin The cells responded with morphological alteration inhibition of macromolecular synthesis and increased permeability Our results are in agreement with these findings

The chain of events which takes place after binding of enterotoxin to the cell membrane is at present unknown Preliminary studies with subcellular distribution of cellassociated enterotoxin in hepatocytes indicate that the enterotoxin exert its effect primarily on the cell membrane In order to reach a final conclusion as to whether the effect on macromolecular synthesis in mammalian cells is a primary effect of internalized toxin or a secondary effect due to membrane damage experiments must be performed in cell free systems

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EFFECTS OF SOME ANTIBACTERIAL AGENTS ON THE PHAGOCYTOSIS OF ^{32}P-LABELLED *ESCHERICHIA COLI* BY HUMAN POLYMORPHONUCLEAR CELLS

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Melby K & Midtvedt T Effects of some antibacterial agents on the phagocytosis of ^{32}P labelled *Escherichia coli* by human polymorphonuclear cells Acta path microbiol scand Sect B 88 103-106 1980

Gentamicin trimethoprim cephalothin colistin erythromycin oxytetracycline and chloramphenicol were studied in a phagocytic system A radiolabelled strain of *E coli* was used as test bacterium and human polymorphonuclear cells were used as phagocytes Except for trimethoprim and cephalothin there was a tendency towards depression of the process of phagocytosis in the presence of high concentrations of the various antibiotics Colistin in a high concentration (83 µg/ml) exerted the most significant effect

Key words Phagocytosis antibiotics human polymorphonuclear cells *E coli*

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8 vii 79 Accepted 8 xi 79

ell known that during an attack by an agent antibacterial drugs only assist the c system in eradicating the invading A possible interaction between the of the phagocytic system and these drugs ved increased attention (1 5 7 8 9 10 present report is an attempt to evaluate the some antibacterial drugs on the process of osis of a radiolabelled strain of *Escherichia* uman polymorphonuclear cells (PMN)

MATERIALS AND METHODS

honuclear cells (PMN) were isolated (4) from ed whole blood (5 I U /ml) obtained from donors by venipuncture and thereafter transfer glass tubes in order to yield a monolayer of es of which approximately 80% were PMN as d previously (14)

m obtained from healthy blood donors was and stored in small aliquots at −80 °C

eria The strain used has been described earlier as a member of the species *E coli* according to Bergey s Manual (3) The strain was stored in lyophilized state at 5 °C and also kept in subculture on lactose bromthymol blue agar plates

Culture technique The medium used was prepared according to *Benacerraf et al* (2) The bacterium was cultivated in 50 ml medium for 18 h Four ml of this culture was transferred to 200 ml of medium to which was added 1 mC ^{32}P labelled orthophosphate The bacterial growth was discontinued after 200 minutes by rapid cooling of the suspension to 0 °C The number of viable bacteria determined as colony forming units (CFU) after dilution and plating was $10^9 \pm 0.5$ log All incubations were kept aerobically in a shaker at 37 °C

P

Conn USA) The bacterial suspension was washed three times with a chilled (4 °C) Krebs Ringer phosphate buffer which was enriched with 10 mM of gl (KRG

USA) After 30 minutes the medium was decanted and a solution of the antibiotic in KRG was added Half an hour later the

medium was decanted again and the monolayers washed twice with prewarmed KRG (37 °C) Samples of 2 5 ml of the bacterial suspension containing 10⁹ ± 0 5 log radiolabelled *E coli* per ml were added and the tubes were left for incubation for 15 minutes at 37 °C The two types of bacterial suspensions used were bacteria suspended in KRG alone and bacteria suspended in KRG containing 10% normal human serum Serum was added immediately before the transfer of the bacterial suspension to the test tubes On termination of the ingestion phase the suspension was decanted and the tubes washed four times with chilled (4 °C) KRG and left for drying In order to dissolve the cells 2 ml of Lowry s Alcaline Copper Solution (LACS) (11) was added when the tubes were dry Thereafter samples of 0 2 ml were removed for determination of radioactivity and 0 5 ml for determination of cell protein content The count per mg cell protein reflects the ingestion of radiolabelled *E coli* The effects of the various drugs were expressed by relating counts per mg cell protein in the untreated tubes to the data obtained from tubes incubated with the various drugs KRG and serum control tubes were always included and all experiments were performed in triplicate

Determination of protein content was carried out according to the method of *Lowry et al* (11) modified by *Oyama & Eagle* (18) Lyophilized human albumin with 96% purity (Sigma Chemical Chemical Comp Mo USA) dissolved in distilled water was used as reference

Determination of radioactivity was performed with aliquots in a Hewlett Packhard liquid scintillation counter

Drugs The antibiotics used and the concentrations applied are shown in Table 1 All drugs were primarily dissolved in distilled water and diluted to the appropriate concentrations using KRG with the exception of oxytetracycline which was dissolved in distilled water and diluted in KRG devoided of Ca + + and Mg + + In the presence of oxytetracycline KRG lacking Ca + + and Mg + + was always used Trimethoprim lactate was dissolved in distilled water to which HCl (4N) was added to give pH 4 0 For complete dissolution the suspension was kept at 37 °C for 15 minutes Further dilutions were carried out with prewarmed (37 °C) KRG In the various concentrations to be tested the pH was neutral

Statistical analysis The Wilcoxon White rank test (12) was used (N 2 P < 0 05)

RESULTS

The effect of seven antibacterial agents on phagocytosis is shown in Table 1 These results indicate that apparently some have an influence on the ingestion phase of phagocytosis when the PMN monolayer has been pretreated with the various antibacterial drugs for 30 minutes before the start of the ingestion period

TABLE 1 *Influence of Some Antibacterial Agen[...] Phagocytosis of ³²P labelled* E coli *by Human P[...] phonuclear Cells* in vitro

Drug	Concentration µg/ml	Per cent a[...] KRG	Se[...]
Cephalotin	30	108	[illegible]
	300	126	[illegible]
Chloramphenicol	10	91	[illegible]
	200	99	[illegible]
Colistin	8 3	100	[illegible]
	83	81b	
Erythromycin	10	93	[illegible]
	100	108	[illegible]
Gentamicin	5	111	[illegible]
	100	114	
Oxytetracycline	10	93	
	100	112	
Trimethoprim	10	106	
	100	104	[illegible]

The results are given as percentages comp[...] untreated controls and are the arithmetic means of [...] observations for each drug
a − p − 0 1 b − p < 0 05 c = p < 0 01

Using high doses of either chloramphen[...] erythromycin in the presence of normal [...] during the uptake of bacteria a trend [...] depression of the phagocytic process was [...]

Using high concentrations of colistin the [...] tive capacity was reduced to 81 % when only [...] was present during the ingestion phase (p< [...] and 76 % when serum was present durin[...] period of ingestion (p < 0 01)

Gentamicin tested with normal serum p[...] during the period of ingestion seemed to imp[...] ingestion phase slightly Using 5 µg/ml as [...] concentration the ingestion was reduced to 8[...] = 0 1) whereas when the concentration [...] increased to 100 µg/ml the uptake of bacteria [...] 80 % of the normal uptake (p = 0 1)

Oxytetracycline in a dose of 100 µg/ml tog[...] with normal serum exerted some influence o[...] uptake of bacteria since only 76 % of the n[...] ingestive capacity was recorded (p = 0 1) [...] difference may be regarded only as a tender[...]

Apparently trimethoprim and cephalothin d[...] influence the process of phagocyt[...]

DISCUSSION

[...]cordance with other reports (5 9 13 17) on [i]nteraction between beta lactam antibiotics and [phag]ocytosis no significant effect was shown when [...] g cephalothin

[Co]listin a polypeptide antibiotic very similar to [poly]myxin B interferes with the cell membrane [funct]ion as a transport barrier of smaller molecules [Unde]r the influence of this antibiotic it has been [demo]nstrated that the cell membrane of bacteria [beco]mes leaky (The effect of this antibiotic has [been] studied on human leukocytes with no measu[rable] effect when using 115 µg/ml (8) Other [work]ers using guinea pig leukocytes and 1200 µg/[ml r]eport about a 22% reduction in the ingestive [capac]ity (5) In our experimental system a depres[sive] effect was found with a test concentration of 83 [µg/m]l The effect seemed to be most pronounced [whe]n normal serum was present during the period [of in]gestion This is partly in accordance with other [repo]rts (13) using rat PMN and the same concentra[tion] of colistin In those studies a depressive effect [was f]ound when 83 µg/ml was used The [depress]ive effect was eliminated by the addition of [...] The reason for this discrepancy is not clear [...]it be due to different affinities of the drug to [var]ious types of cells and sera Thus high doses [of coli]stin seem to impair the process of ingestion [...] phagocytosis as shown in our study

[Seve]ral studies have shown that high concentra[tions o]f chloramphenicol inhibits the process of [phagoc]ytosis (6 10) Using 200 µg/ml of this [antibio]tic it has previously been demonstrated that

[...] results presented also indicate inhibitory [...] on the phagocytic process by gentamicin This [contra]dicts previous findings (9 13)

[Fe]w reports are available regarding the effect of [tetrac]yclines on the process of phagocytosis *In vivo* [Altur]a et al (1) have demonstrated that administra[tion o]f 12 mg per day in drinking water for four [...] decreased the efficiency of the phagocytic [syste]m of rats to clear carbon particles injected into [the s]ystemic circulation *Munoz & Geisler* (15) [...]ted inhibition of the engulfment of a micrococ[cus s]train by the action of chlortetracycline applied [to t]he human leucocytes tested in an *in vitro* [syste]m The lowest effective concentration found [was] 0 01 µg/ml *Forsgren et al* (7) have extended [these] findings by their studies on *E coli* and yeast [...] presented to human PMN together with [...]cycline or tetracycline A decreased precentage of leucocytes containing microorganisms could be demonstrated with either of these antibiotic in the range of 2 5–25 µg/ml Other studies (8 9) do not confirm these findings even with high concentrations of tetracycline (426 µg/ml) The results presented in this study support the opinion (1 7 13 15) that the tetracyclines apparently have an inhibitory influence on the process of phagocytosis However the concentrations necessary to exert such influence seem to be rather high though not higher than what can be obtained in the regions of the human body where the drugs are concentrated (6 16) Our findings support the results obtained from studies on rat PMN (13)

To our knowledge there is no other report dealing with the interaction of trimethoprim and the phagocytic system except our previous studies using rat PMN (13) This compound seems to have no influence on the ingestion phase of the phagocytic process

It is likely that some antibiotics do have an influence on the process of ingestion when a microbe is to be phagocytosed by PMN The concentrations apparently necessary to reveal these effects are obtainable in the human body (16) The clinical implications of these findings are difficult to establish The reduced effect on the uptake of bacteria shown with high doses of colistin is worthy of attention

It may be concluded that except for trimethoprim and cephalothin there is a tendency towards a depression of the ingestion phase when high concentrations of the other antibiotics tested are applied

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EXPERIMENTAL SALPINGITIS IN GRIVET MONKEYS BY *CHLAMYDIA TRACHOMATIS*

Modes of Spread of Infection to the Fallopian Tubes

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Møller B R & Mårdh P A Experimental salpingitis in grivet monkeys by *Chlamydia trachomatis* Modes of spread of infection to the Fallopian tubes Acta path microbiol scand Sect B 88 107–114 1980

Experimental infection with *Chlamydia trachomatis* immunotype K was carried out in six grivet monkeys Three routes of infection were used (A) into the uterine cavity through the cervical canal (B) into the uterine cavity but after ligature of the tubal isthmus followed by curettement of the endometrium (C) into the cervical epithelium Two monkeys were used *for each type of experiment* All monkeys inoculated by routes (A) and (C) developed marked signs of inflammation in the Fallopian tubes Characteristically the tubes were reddened and swollen Exudate appeared in the abdominal ostia and *in the cul-de sac In three* of the four monkeys studied a yellowish discharge was seen in the posterior vaginal *fornix An exudate* composed mostly of lymphocytes and clusters of desquamated cells was *found in the lumen* of the tubes The epithelium was injured in some areas and there were adhesions between [illegible] keys tubal occlusion was present two months [illegible] presented a significant rise in antibodies to C [illegible] no signs of inflammation in the upper genital tract were seen but one of the *animals died day 14* post inoculation autopsy showed peritonitis and perihepatitis One monkey was inoculated with non infected *yolk* sac specimen by route (A) and another by route (B) Practically no inflammatory *changes in the genital organs* or elsewhere could be detected in these monkeys The results indicate that *C trachomatis* can cause salpingitis in female *grivet* monkeys by *canalicular spread from* the lower genital tract The histological findings and the infection route in this experimentally induced salpingitis in the grivet monkeys closely resemble those ascribed to gonococcal salpingitis in man

Key words *Chlamydia trachomatis* salpingitis experimental infection

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[C]hlamydia trachomatis is a common cause of [sexu]ally transmitted diseases (8 9 12) In the [fem]ale the organism may produce cervical infection [asso]ciated with abnormal Papanicolaou smears (1) [Fro]m the cervix, chlamydiae may reach the tubal [epit]helium and cause acute salpingitis (6) However, [the] mode of spread of the infection from the cervix [to t]he tubes is not known

[I]n a previous study (11) *C trachomatis* was [inoc]ulated [illegible] tubes or into the uterine cavity of grivet monkeys Within a few days these monkeys developed salpingitis However this study did not confirm the route of infection to the *tubes* neither did it simulate conditions *comparable* to those of naturally infections in the *female*

Experiments in grivet monkeys were therefore designed to mimic conditions that might occur in *natural* infections with chlamydiae in the female, *viz.* the opportunity for a canalicular spread of such organisms *through the* uterine cavity to the lumen

of the Fallopian tubes and/or spread via lynphatics and blood vessels through lesions in the cervical epithelium or in the uterine endometrium The results of these studies are given in the present communication

MATERIAL AND METHODS

Organism A strain of *C trachomatis* immunotype K was isolated from the Fallopian tubes of a patient with acute salpingitis This strain was used to infect a grivet monkey into the uterine tubes as described previously (11) This monkey developed acute salpingitis In the present study the strain isolated from the patient (No 771) and that (No 5155) reisolated from the cervix of the infected monkey were used The strains had been isolated on cycloheximide treated McCoy cells (10) and passed twice in the yolk sac of embryonated hen eggs Suspensions of the yolk sac material containing 2×10^5 inclusion forming units per ml were stored at −80 °C until use A hundred fold dilution of the suspension in phosphate buffered saline pH 7 2 was used to inoculate the monkeys

A yolk sac suspension from a non infected embryonated hen egg diluted in PBS as described above was also tested

Monkeys Eight female grivet monkeys *(Cercopithecus aethiops)* were used in the experiment They weighed 2 0–2 6 kg They had been captured in East Africa and before use had been kept in quarantine for at least six weeks They were examined and caged as described elsewhere (5)

Specimens were collected on three consecutive occasions from the throat the urethra the cervix and the rectum and cultured for *C trachomatis* mycoplasmas and ureaplasmas The sampling and culture techniques used were those described elsewhere (11) Blood samples were taken by puncture of the femoral artery in the inguinal region All samples were collected when the monkeys were anaesthetized using ketamine chloride (Ketalar® 5 mg/kg i m)

Experimental infections The monkeys were infected in three different ways

(A) In monkeys I and II 0 4 ml of the suspension of chlamydiae (strain No 771) was injected into the uterine cavity using a steel catheter 0 4 mm in diameter introduced through the cervical canal

(B) In monkeys III and VI the isthmus of the Fallopian tubes was closed by ligatures during laparotomy performed as described elsewhere (5) In these latter monkeys 0 4 ml of chlamydial suspension (strain No 771) was injected as described in experiment (A) The cervix was then dilated to 1 5 mm using modified ... C curettage of the uterine endometrium ... 1 2 mm ...ension of

Monkey VII was inoculated with ...

sac specimen in the same way as in experim... Monkey VIII was infected as in experiment (B)...

Assessment of lesions and collection of sp... Laparotomy was performed on days 4 7 21 3... post inoculation (p i) At each laparotomy biop... taken from the Fallopian tubes and parametr... mens collected by a swab were taken from t... sites the tubal fimbriae the cul de sac and the...

Culture studies Cultures for C trachomatis... mycoplasmas (5) were performed as described e... Bacterial cultures were made in brain heart... broth (Difco) and on blood haematin and... bromthymol blue agar

Serological studies Surveys for serum anti... *C trachomatis* were carried out by a micro imm... rescence test using pools of antigen (13) The... titrated in two fold dilutions The lowest dilu... was 1 8 All sera were tested for both IgG... antibodies to the organism

Histological studies Tissue biopsies were... 10% formalin and paraffin embedded for hi... studies using staining with haematoxyl n and...

RESULTS

General condition of the infected monkeys ... eight monkeys only one presented any clin... of disease Thus monkey III developed fa... showed slow and prudent movements da... and loss of appetite The illness progresse... monkey died day 14 p i The monkey... incapable of eating and drinking for... previously

Gross lesions In monkeys I II V an... inflammatory lesions were practically ide... days 7 14 and 21 p i the tubes were red... swollen Exudate appeared in the abdomin... the tubes and a watery exudate was found i... de sac Day 35 p i the inflammatory rea... regressed On day 60p i the appearance of... was virtually normal No gross lesions w... in the parametria the ovaries and th... throughout the experimental period Othe... ritoneal organs including the spleen... macroscopical signs of inflammation

Monkey III presented no gross lesions... and 7 p i in the tubes the parametria the... the spleen There was no exudate in the... ostia of the tubes or in the cul-de sac Th... died on day 14 p i Autopsy showe... peritonitis and perihepatitis with adhesion... the peritoneum and the anterior surface of... The spleen was enlarged The intern... organs and the intestine were normal Th... no signs of pneumonia







... Histological section of the Fallopian tube of ...ey II inoculated into the uterine cavity with *C ...omatis* immunotype K day 4 post inoculation ...ing inflammatory infiltrate in the tubal epithelium ...n the subepithelial tissue HE × 50

...IV and in the control monkeys (VII and VIII) ...ughout the experimental period

...yellowish discharge was seen in the posterior ...*nal* fornix of monkeys II V and VI from day 7 ...and during the following 2 to 3 weeks There ...e no erosions of the cervix

...istological findings Monkeys I II V and VI all ...ented histological inflammatory changes in the ...er genital tract The alterations were pronounced

... 2 Histological sections of the Fallopian tube of ...key VI inoculated into the cervical epithelium with ...*trachomatis* immunotype K (A) On day 7 post ...ulation pronounced inflammatory infiltrate in the ...al epithelium and in the subepithelial tissue can be ...n The epithelium is injured in some areas HE × 60 ... On day 14 post [illegible] ... [illegible] ... [illegible] ... [illegible] ...ammatory cells mostly lymphocytes HE ...0

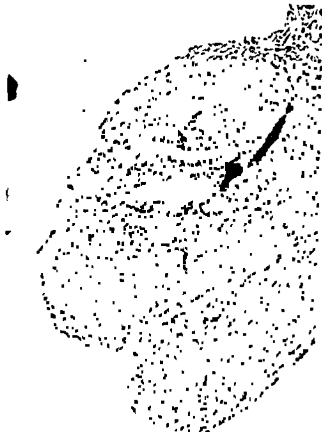


Fig 3 Histological section of the Fallopian tube of monkey V inoculated into the cervical epithelium with *C trachomatis* immunotype K day 60 post inoculation Adhesions between the mucosal folds and infiltration of a small number of inflammatory cells in the subepithelial tissue and the muscular layers can be seen HE ×20



Fig 4 Histological section of the Fallopian tu monkey VI, inoculated into the cervical epithelium *C trachomatis* immunotype K, day 60 post inocu The tubal epithelium is atrophic and flattened the lumen diminished and the muscular layers incre thickness HE ×15

in monkeys II, V and VI, but less so in monkey I In monkey I, the tubal epithelium and the subepithelial tissue showed infiltration with lymphocytes and some polymorphonuclear leukocytes on days 7 14 and 21 p i The tubal epithelium was intact and there was no inflammation in the tubal muscular layers in the subserosa or in the parametria On days 35 and 60 p i , practically no histological signs of inflammation were found

In monkeys II V and VI, there was infiltration in the tubal epithelium and the subepithelial tissue with abundant lymphocytes and some leukocytes on day 4 p i (Fig 1) On days 7, 14 and 21 p i , the lumen of the Fallopian tubes contained an exudate with lymphocytes and clusters of desquamated cells The epithelium was injured in some areas and a few adhesions were seen between the mucosal folds (Figs 2A and B) On day 35 p i , a moderate inflammatory reaction was found in the epithelium and in the subepithelial tissue, but the epithelial lining of the tubes was practically intact Adhe between the mucosal folds were present in mo V and VI On day 60 p i , the tubes of monk were normal In monkey V, the mucosal folds thickened and adhesions were still present a number of inflammatory cells were seen i subepithelial tissue In monkey VI, the lumen tubes was diminished and the tubal epithelium atrophic and flattened The muscular layers increased and inflammatory cells were pres the subepithelial tissue The parametria re only minimal inflammatory infiltration, most the subserosa (Figs 3 and 4)

Monkey III presented no signs of inflamm in the tubes or parametria on days 4 and 7 Autopsy on day 14 p i showed slight inflamm infiltration of the subserosa of the Fallopian t while the muscular layers, the subepithelial t and the epithelium were normal Moderate inf tion of inflammatory cells was



His olog cal section of the parametria of monkey
:ulated into the cavum uteri after l gat on of the
thmus with *C trachomat s* immunotype K day
. inoculation Moderate nf ltration of inflamma
lls particularly around the blood vessels can be
E × 120

:tria, particularly around the blood vessels
) There was no inflammation in histological
s of the portio the cervical canal the uterus
: ovaries Sections from the liver showed

as enlarged and a great number of
cytes polymorphonuclear leukocytes and
lasma cells were seen No signs of infection
und

onkey IV on day 4 p i slight inflammatory
iltration was found in the parametria but
as no oedema On day 7 p i a mode

s of n ... p i there was

though very slight were confined to the subserosa of the Fallopian tubes and the parametria

Haematological find ngs Monkeys III and IV showed a pronounced rise in the erythrocyte sedimentation rate (ESR) during the first week p i viz from 3 to 52 mm/h and from 2 to 60 mm/h respectively In the same monkeys there was a marked rise in the leykocyte count (LC) viz. from 8 100 to 17 300/µl and from 6 700 to 10 600/µl The other six monkeys studied showed no or only slight changes in the ESR and the LC after the infection (Table 1)

Isolation of C trachomatis Chlamydiae could not be recovered from monkey I during the investigation period In monkey II the organism was isolated from the tubes and from the cul-de-sac on day 21 p i In monkey III *C trachomatis* was isolated from



istolog cal section of the l ver of monkey III
inoculated nto the uter ne cav ty af er

TABLE 1 *Erythrocyte Sedimentation Rate (ESR) and Leukocyte Count (LC)×10³ in Grivet Monkeys I*
Experimentally with C. trachomatis *Immunotype K (Monkeys I–VI) and in Two Control Monkeys (VII a.d.*

Site of inoculation	Monkey No		Days post inoculation 0	4	7	14	21	35
Uterus	I	ESR	ND[a]	5	8	4	2	ND
		LC	ND	5 8	5 8	6 6	ND	ND
	II	ESR	2	8	7	1	3	2
		LC	6 9	6 2	8 0	7 2	6 5	9 2
Uterus (followed by curettement)	III	ESR	3	ND	52	ND[b]		
		LC	8 1	ND	17 3	9 0		
	IV	ESR	2	60	50	17	2	1
		LC	ND	6 7	10 6	9 4	9 1	3 9
Cervix	V	ESR	2	2	20	16	2	ND
		LC	8 1	6 3	9 1	6 8	6 5	ND
	VI	ESR	1	2	3	3	2	ND
		LC	5 4	5 8	4 6	4 9	6 4	ND
Controls	VII	ESR	2	2	ND	4	4	ND
		LC	7 5	7 0	ND	6 8	6 6	ND
	VIII	ESR	3	5	4	4	ND	ND
		LC	6 2	6 2	6 8	6 7	ND	ND

[a] ND – not done
[b] Monkey III died on day 14 p.i.

the cervix on days 7 and 14 p.i. and from the uterine cavity on day 14 p.i. Samples collected from the tubes and the fimbriae revealed no growth of chlamydia nor did samples collected from the surface of the spleen, the instestine and from the lungs. In monkeys IV, V and VI chlamydiae were isolated days 14, 21 and 7 p.i. respectively.

From the control monkeys, viz. monkeys VII and VIII, no chlamydiae could be isolated on any of the sampling occasions.

No mycoplasmas were isolated from the specimens of the eight monkeys collected in connection with the laparotomies. *With the exception of sparse* growth of coagulase positive staphylococci isolated from the fimbriae and cul de sac day 21 p.i. of monkey II and of coagulase negative staphylococci from the tubes of monkey IV day 7 p.i., the bacterial cultures were negative.

Antibody studies. No antibodies to *C. trachomatis* were detected in serum drawn before the inoculations. No IgM or IgG antibodies to *C. trachomatis* were detected during the investigation period in the serum samples from monkeys III (which died day 14 p.i.) and V or from the two control mo[…] IgM antibodies were detected in monkeys II a[…] while IgG antibodies were found in monkey[…] IV and VI (Table 2).

DISCUSSION

Salpingitis may be caused by an infection from adjacent organs, generally an inflamed [appen]dix, or, as in the vast majority of cases, infection ascending from the lower genital tr[act] *principle two groups of ascending infections* recognized. One type is the so-called ›gonoc[occal] salpingitis in which the causative organism [reaches] the tubes from the cervix by canalicular sprea[d via] the uterine mucosa to the tubes. This ty[pe of] salpingitis produces a suppurative inflammat[ion of] the endosalpinx that may lead to tubal occl[usion]. The parametria are usually not affected. I[n the] › non gonococcal type of salpingitis the orga[nisms] are believed to invade the tissue through lesi[ons of] *the cervical epithelium or of the ute[…]*

2 *Titres of IgM and IgG Antibodies to C ...matis in Grivet Monkeys Infected Experimentally ...he Organism and Control Monkeys (VII and VIII)*

...ey No		Days post inoculation				
		7	14	21	35	60
I	IgM	–	–	–	–	
	IgG	–	–	–	64	–
II	IgM	–	64	32	–	–
	IgG	–	–	128	512	128
II	IgM	–	–*			
	IgG	–	–			
V	IgM	–	–	–	–	
	IgG	–	–	128	128	64
V	IgM	–	–	–	–	–
	IgG	–	–	–	–	16
VI	IgM	–	–	16	–	–
	IgG	–	–	16	1024	512
II	IgM	–	–	–	–	–
	IgG	–	–	–	–	–
III	IgM	–	–	–	–	
	IgG	–	–	–	–	–

...onkey III died on day 14 p i

...um and are spread via lymphatics and blood ...ssels to the tubes parametria and broad liga...ents In this latter type of salpingitis pronounced ...flammation occurs in the parametria The inflam...ation in the tubes is generally confined to the ...ibserosa and the endosalpinx is usually unaffected

Besides being caused by *Neisseria gonorrhoeae* e gonococci ...

...istopathological picture similar to that found in ...hese latter monkeys was demonstrated in two ...atients with pyosalpinx caused by *C trachomatis* ...6)

The present study seems to indicate that chlamy...iae may spread canalicularly from the cervix to the ...pper genital tracy When the isthmus of the tubes ...as closed by ligatures as was the case in monkeys II and IV no inflammation of the tubes or ...arametria occurred However these two monkeys ...resented a marked rise in the ESR and the LC

Monkey III died on day 14 p i Autopsy of this ...onkey showed peritonitis and enlargement of the spleen Perihepatitis also occurred Acute fibrinous perihepatitis reported already nearly fifty years ago by *Fitz Hugh* (4) and *Curtis* (2) has been described as a complication of gonorrhoea Recently *Muller Schoop and co workers* (7) reported peritonitis and perihepatitis in young women with high antibody titres to *C trachomatis*

Autopsy of monkey III revealed no evidence of a generalized infection although the spread of *C trachomatis* from the uterine cavity to the abdominal organs probably occurred via blood vessels and lymphatics Canalicular spread was not possible because of the ligatures

In 1946 *Falk* (3) reported on cornual resection in more than 1 000 women with infected Fallopian tubes The patients were followed up for more than eight years and not one single case of recurrent salpingitis was found *Falk* concluded that the cornual resections prevented canalicular spread from the cavum uteri to the tubes and protected against ascending organisms The author suggested that in most cases of primary salpingitis gonococci are the causative agents and that such organisms may ascend from the lower genital tract through the uterus to the tubes However he was only able to recover gonococci from the tubes of these patients in a few cases Chlamydial infection was not included in that material In the present study we found that ligation of the tubal isthmus prevented *C trachomatis* from reaching the tubal epithelium and causing salpingitis *N gonorrhoae* and *C trachomatis* both induce salpingitis which is very similar in histopathological appearance Also the method of spread of the organism to the tubes is the same

Sequelae of salpingitis in the female is known to cause impaired fertility and sterility due to obstruction of the lumen of the tubes In the present study *C trachomatis* produced inflammation in the tubes of four monkeys In two to them (monkeys I and II) the tubal epithelium was intact day 60 p i and there were no inflammatory reactions However in monkeys V and VI the epithelium of the tubes was still not normalized on day 60 p i Thus in monkey V the mucosal folds were thickened and adhesions between the folds were seen while in monkey VI the lumen was reduced in diameter and the epithelium was atrophic and flattened Such changes may impede transport of a fertilized ovum from the ovary to the uterus thereby impairing fertility or causing sterility and an increases risk of ectopic pregnancy (14)

The present study showed that in grivet monkeys *C trachomatis* is able to attack the tubal epithelium and produce salpingitis The infection may reach the tubes via canalicular spread from the cervix In some of the monkeys studied partial

TABLE 1 *Bacterial Strains Examined*

F necrophorum	VPI 6161 SPH 1 N 167
F mortiferum	VPI 0473 VPI 4249 VPI 5696
F gonidiaformans	VPI 0482A VPI 4381 VPI 4879 VPI 11360
F varium	ATCC 8501 VPI 0499A VPI 4234 Lille 109
F naviforme	VPI 4877 VPI 11936C VPI 12188
F russi	VPI 0307 VPI 10646 VPI 11018
F plauti	VPI 0310 VPI 0311 VPI 0312
F nucleatum	F1 F3 F5 F6 F7 F9 F10 F11 F14 F18

VPI Virginia Polytechnic Institute and State University Blacksburg Virginia USA
ATCC American Type Culture Collection Rockville Rockville Maryland USA
Lille Institute Pasteur de Lille Lille France
Strains SPH 1 and N 167 were received from Dr *Justesen* Copenhagen Denmark and Dr *Beerens* Lille France respectively
The *F nucleatum* strains were own isolates

MATERIALS AND METHODS

Microorganisms

The bacterial strains examined are listed in Table 1 They were cultivated in an enriched fluid medium based on tryptone (1) After harvesting by centrifugation the cells were washed twice in saline and stored as a paste at −20 °C until used

Isolation of LPS

Frozen cells (1 g) were treated with constant stirring with 45 per cent aqueous phenol (35) (5 ml) at 20 °C for 30 min The LPS were purified from the water phase by ultracentrifugation (100 000 × *g* for 90 min) and by treatment of the pellet with ribonuclease and deoxyribonuclease (8)

Gas Liquid Chromatography (GLC)

For the determination of neutral sugars samples of LPS were hydrolysed in sealed ampoules with 0 1 N HCl for 48 h at 100 °C The hydrolysed samples were neutralized with Amberlite IRA 410 HCO_3 form and the aldoses converted to alditol acetates (24) GLC was performed by a Perkin Elmer 900 gas chromatograph

USA) The carrier gas (N_2) flow was 30 ml per min and the injector and detector temperatures were 200 and 250 °C respectively whereas the column was programmed from 180 to 240 °C at a rate of 2 °C per min D *xylose* was used as an internal standard The gas chromatographic peaks were tentatively identified by comparing retention times with those of standards including a hydrolysate of a *Salmonella typhimurium*

LPS which contains both L and D D *manno*heptose (20) (obtained from D 0 L Freiburg FRG)

For analysis of fatty acid composition LPS (0 5 to 1 5 mg) were methanolysed with methanol (1 5 ml) in teflon lined screw-cap 85 °C for 18 h (13) The fatty acid methyl extracted with hexane and treated with anhydride/acetonitrile (14) before GLC on a column packed with 3 8 per cent SE 60–80 mesh (Serva Heidelberg FRG) The temperature was programmed from 1?0 to ? rate of 2 °C per min Otherwise the conditions were as described above As internal *n* heptadecanoic acid was used A NHID Mixture (Supelco Inc) 3 hydroxy tetradecan 3 hydroxy hexadecanoic acid (Applied Science Laboratories Inc State College Pennsylvania USA) as external reference standards

Paper Chromatography

For identification of amino sugars samples were first hydrolysed with 2 N HCl at 100 °C Acid was removed from the hydrolysates by in *in vacuo* over NaOH pellets Circular paper chromatography was done using Whatman No 1 paper solvent system butan 1 ol pyridine water vol) Detection reagents used were ninhydrin alkaline silver nitrate (28)

Colorimetric Determination of KDO

The presence of KDO was examined by the thiobarbituric acid method (33) Samples of LPS were hydrolysed with 0 02 N H_2SO_4 for 20 min at 100 °C The density was examined in the range of 500 to 600 a peak at 550 nm was recorded as a positive

RESULTS

LPS were obtained in varying yields from strains listed in Table 2 and Table 3 The co neutral sugars and fatty acids accounted for per cent of the dry weight of the prepa When different batches of LPS from the sam were examined the total amount of neutral varied somewhat but the molar ratios different sugar constituents were similar

Heptose I which had a retention time like that of L *glycero* D *manno*heptose was in all LPS as a main sugar component (T particularly so in the LPS isolated from the of *F gonidiaformans* Galactose was found LPS preparations examined except those strains VPI 4381 VPI 4234 and Lill Glucose could not be detected in *F necro* SPH 1 *F mortiferum* VPI 5696 and *F* strain VPI 0499A but was otherwi present

- E 2 *Neutral Sugars in LPS from* F necrophorum F mortiferum F gonidiaformans F varium F naviforme *Strain VPI 4877 and* F russi *Strain VPI 0307*

r 1	LPS yield	Neutral sugars (%)	Molar ratios of				
			Heptose I	Heptose II	Glucose	Galactose	Rhamnose
rophorum							
PI 6161	1 45	10 2	1		tr	1 6	
PH 1	1 31	17 8	1			2 5	
167	2 53	11 5	1		0 3	1 3	
ortiferum							
PI 0473	2 16	22 3	1	0 3	0 4	0 5	
PI 4249	6 16	25 9	1	0 4	0 4	0 3	
PI 5696	5 48	10 5	1			1 0	0 8
onidiaformans							
PI 0482A	1 75	18 8	1	0 1	0 3	0 2	
PI 4381	1 72	22 6	1	0 1	tr		
PI 4879	1 66	14 4	1	0 1	tr	0 1	
PI 11360	1 00	18 1	1	0 1	0 2	0 2	
arium							
ATCC 8501	3 50	7 6	1	0 1	0 8	1 0	
VPI 0499A	3 53	20 3	1			0 7	
VPI 4234	3 27	31 9	1	0 7	0 6		
ille 109	0 82	14 5	1	0 8	0 8		
a iforme							
VPI 4877	1 73	25 2	1		0 5	0 2	
russi							
VPI 0307	1 18	11 8	1		0 4	1 0	0 9

a Mg freeze-dried LPS obtained from 1 g of wet bacteria
trace amounts
ptose I retention time GLC similar to L *glycero*-D *manno*heptose
ptose II retention time GLC similar to D *glycero* D *manno*heptose

Paper chromatography revealed the presence in ll LPS of glucosamine Galactosamine was not etected Most preparations contained ninhydrin ositive components with a faster chromatographic obility than glucosamine As these compounds ere silver nitrate negative (not reducing) their ature was not examined

Small amounts (less than 1 per cent) of KDO were present in all LPS

All LPS preparations examined contained *n* tetradecanoic acid and 3 hydroxy tetradecanoic acid (Table 3) The LPS isolated from strains of *F nucleatum* contained in addition *n* hexadecanoic and 3 hydroxy hexadecanoic acid The former constitu ent was also present in LPS prepared from *F necrophorum* SPH 1 *F russi* VPI 0307 and the strains of *F mortiferum*

Only small amounts of a non gelatinous material were obtained from the strains of *F plauti* and the VPI strains 11018 11936C and 12188 (Table 1) These preparations did not contain the LPS markers 3 hydroxy tetradecanoic acid and heptose GLC analysis indicated only small or minute amounts of

detected

TABLE 3 *Fatty Acids[a] in LPS from* F necrophorum F mortiferum F gonidiaformans F varium F navif *Strain VPI 4877* F russi *Strain VPI 0307 and* F nucleatum

Strain	Fatty acids (%)	Mol % of			
		14 0	3 OH 14 0	16 0	3 OH 16 0
F necrophorum					
VPI 6161	10 4	23 4	76 6		
SPH 1	16 3	25 3	61 3	13 4	
N 167	9 4	22 9	77 1		
F mortiferum					
VPI 0473	26 4	19 1	65 6	15 3	
VPI 4249	13 8	26 4	47 2	26 4	
VPI 5695	14 9	17 7	66 2	16 1	
F gonidiaformans					
VPI 0482A	10 1	35 3	64 7		
VPI 4381	11 1	31 4	68 6		
VPI 4879	18 6	17 6	82 4		
VPI 11360	13 4	24 0	76 0		
F varium					
ATCC 8501	11 1	23 5	76 5		
VPI 0499A	16 1	31 1	68 7		
VPI 4234	19 0	22 5	77 5		
Lille 109	9 6	31 8	68 2		
F naviforme					
VPI 4877	9 1	21 1	78 9		
F russi					
VPI 0307	14 0	17 4	68 1	14 5	
F nucleatum					
F5 F7 F10 F14 F18	6 4–15 2	33 4 37 0	38 5–40 7		23 9 26 9
F1 F3 F6 F9 F11	10 6–17 6	22 7–35 6	28 9–43 9	6 1–17 7	17 9 27 5

a) Some preparations contained 16 0 and a few 12 0 in trace amounts ($<1\%$ dry weight)
14 0 *n* tetradecanoic acid 3 OH 14 0 3 hydroxy tetradecanoic acid
16 0 *n* hexadecanoic acid 3 OH 16 0 3 hydroxy hexadecanoic acid

DISCUSSION

The presence of the typical LPS constituents 3 hydroxy tetradecanoic acid KDO and heptose characterized the LPS preparations from *F necrophorum F mortiferum F gonidiaformans F varium F nucleatum* (see also 1) *F naviforme* VPI 4877 and *F russi* VPI 0307 On the other hand no constituents typical of LPS could be detected in the extracted materials of the three strains of *F plauti* *F naviforme* strains VPI 10646 and VPI 12188 and *F russi* strains VPI 10646 and VPI 11018 It is of relevance that the fatty acid patterns of whole cells of the same bacterial strains did not incl hydroxy tetradecanoic acid or other known h fatty acids (12) The results indicate that particular strains of Gram negative bacteria be devoid of LPS or more likely the LPS is a with respect to chemical composition This t mically important aspect merits further using other extraction methods

The neutral sugars found in the present L *glycero* D *manno*heptose glucose galactos rhamnose are common constituents of ba LPS D *glycero* D *manno*heptose (hep study) is less widespread and has

F nucleatum (1) Proteus mirabilis (16) vulgaris (25) Yersinia pestis (3) Yersinia olitica (29) Veillonella (6) and as part of soluble LPS of some phototrophic bacteria 32) The sugar is apparently an intermedi the synthesis of L glycero-D mannoheptose nd it has been shown that L- and D glycero-nnoheptose may be part of the same LPS molecule (7 15) Thus the LPS core struc f Rhodospirillum tenue contains three molecu L glycero-D mannoheptose and one molecule glycero-D mannoheptose (Rad iejewska Le personal communication 1979)

important finding was the occurrence in the nt LPS of L glycero-D mannoheptose (hep-) as a major component Within Enterobacte e this is characteristic of rough mutants ver the LPS of F nucleatum strain Fev 1 has ero-D mannoheptose as part of both the accharide core structure and the O specific accharide (7)

Hydroxy tetradecanoic acid is a common cons it of bacterial LPS (21 36) The presence of n decanoic acid as the only non hydroxy fatty in significant amounts has been reported for isolated from Pseudomonas diminuta (37) The matographic examination of the F nucleatum confirmed the previous findings of 3 hydroxy decanoic acid as a taxonomic marker of this nism (4 12) 3 Hydroxy hexadecanoic acid has been f

he LPS of F mortiferum VPI 0473 and F um ATCC 8501 had higher contents of 3 roxy tetradecanoic acid than reported earlier (4) reason is possibly that alkaline hydrolysis ch gives low yields of hydroxy fatty acids (14) used in the former study

he results of the present study indicate that the icture of F

species examined do not seem differ markedly in their chemical composition

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IMMUNOCHEMICAL ANALYSIS OF THE TEICHOIC ACID FROM *STAPHYLOCOCCUS SIMULANS*

ARVE OSLAND ARNE GROV* and PER OEDING

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sland A Grov A & Oeding P Immunochemical analysis of the teichoic acid from *Staphylococcus mulans* Acta path microbiol scand Sect B 88 121-123 1980

he wall teichoic acid of *Staphylococcus simulans* has been characterized as a glycerol phosphate olymer with glycosidically linked N acetylglucosamine Susceptibility to β-N acetylglucosaminidase nd serological similarity to poly C_β from *Staphylococcus saprophyticus* showed that the amino sugar ; in the β-configuration

Key words *Staphylococcus simulans* teichoic acid immunochemistry

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ved 12 xi 79 Accepted 4 xii 79

an skin The species is coagulase negative rt from the statement that *S simulans* teichoic contains glycerol and galactosamine, no data been given on this teichoic acid More wledge of the teichoic acid is desirable particu because an overlap has been reported of several perties of *S simulans* with those of human ins of *S aureus* (4)

MATERIALS AND METHODS

ains

The type strain *Staphylococcus simulans* CCM 2705 TCC 27848) ...

...ong those described in (4) were supplied by W E oos (Raleigh USA) Bacteria of these 9 strains were ed in agar precipitation experiments

Standard Teichoic Acids (Polysaccharides)

Poly C_α (α N acetylglucosaminyl glycerol teichoic acid) from *S hyicus* VA 308 and poly C_β (β-N acetylglucosaminyl glycerol teichoic acid) from *S saprophyticus* 3519 were produced according to (2 7)

Isolation of Polysaccharide

The polysaccharide from *S simulans* CCM 2705 (poly 2705) was extracted from whole bacteria using 70 mM phosphate buffer pH 6 5 at 37 °C Three extractions each lasting 24 h were performed Acid and ethanol precipitation as well as purification on DEAE cellulose (Whatman Ltd England) and Sephadex G 100 (Pharmacia Sweden) columns were performed as described (7)

Qualitative and Quantitative Analyses

Samples (2-5 mg) of polysaccharide were hydrolysed with 0 5 ml 3N HCl for 3 h at 100 °C or 6N HCl for 20 h at 105 °C in sealed tubes flushed with nitrogen Circular paper chromatography was carried out with the following solvent systems

A Propanol NH_3 H_2O (6 3 1 v/v)

B Ethylacetate pyridine H_2O (40 11 6 v/v)

C Butanol HAc H_2O (4 1 1 v/v)

The examination of sugars and sugar alcohols as acetylated deriva...

100/120 mesh (Supelco Inc USA) Amino acids were

* Deceased June 26th 1979

determined quantitatively as trifluoro acetylated butyl esters (8) with a glass column packed with Tabsorb (Regis Chemical Co USA)

Hexosamines were determined quantitatively by a modified Elson Morgan method (3) and phosphorus as described in (10)

Enzymatic Treatment

Polysaccharides (0 05 mg/ml) were treated with 0 03 units/ml β N acetylglucosaminidase (Boehringer BRD) in 100 mM sodium citrate buffer pH 4 4 at 37 °C for 40 h (7) After treatment the reaction mixture was neutralized and then subjected to double diffusion

Serological Methods

Immune sera were raised against formalin killed bacteria by intravenous injections into New Zealand white rabbits(5) Double diffusion in agar was performed as previously described (1) Reaction between polysaccharide and concanavalin A (Con A) (Sigma Chemical Co USA) was carried out as described in (7)

RESULTS

The polysaccharide was eluted from the DEAE cellulose at a molarity of KCl between 0 37 M and 0 42 M Approximately 100 mg of purified polysaccharide was obtained from 100 g of wet bacteria

Paper chromatography of 3N HCl hydrolysates of the purified polysaccharide in solvent system A showed the presence of glycerol in addition to glycerol 1 phosphate using periodate benzidine as detecting reagent No ribitol could be detected Chromatograms sprayed with molybdate reagent showed only two spots one corresponding to glycerol 1 phosphate the other to glycerol diphosphate

Paper chromatography of 3N HCl hydrolysates in solvent system B and C showed the presence of an amino sugar as detected by the silver nitrate and ninhydrin reagents The amino sugar was identified as glucosamine by gas liquid chromatography No galactosamine could be detected The amounts of phosphorus and N acetylglucosamine estimated by colorimetric analyses were 6 3 per cent and 10 3 per cent respectively the molar ratio of N acetylglucosamine to phosphorus thus being 0 23 to 1

Quantitative analysis of amino acids by gas liquid chromatography showed the presence of the usual peptidoglycan amino acids *viz* alanine glycine glutamic acid and lysine the amounts being 0 07 0 09 0 08 and 0 04 µmoles/mg respectively

Antiserum to *S simulans* CCM 2705 bacteria produced one precipitation line against purified poly 2705 This line showed a reaction of identity with one of the several lines produced against CCM bacteria

Antiserum to *S saprophyticus* 3519 precipitation line against poly 2705 wh completely with the line produced by poly C crossed the antiserum/poly 2705 line No l observed between antiserum 2705 and poly C was any line observed between antiserum *hyicus* VA 308 and poly 2705

Treatment of poly 2705 with β N acetylg minidase destroyed its serological activity a antiserum 3519 but not against antiserum The enzyme destroyed the serological act poly C_β but not that of poly C_α

Poly 2705 and poly C_β produced no line a Con A whereas poly C_α did

Antiserum 3519 produced a precipitation *fusing with that of poly C_β with* bacteria CCM 2705 and 7 of the 9 additional *S s* strains tested

DISCUSSION

The teichoic acid from *S simulans* CCM 2 0 N acetylglucosaminyl glycerol teichoic acid indicated by the demonstration of glycerol phate glycerol diphosphate and glucosam reported demonstration of galactosamine teichoic acid of this strain (4) is not in acco with our results Glucosamine was the only sugar present in the poly 2705 preparat results showed that the glucosamine is β glyc ally linked Both the susceptibility to β glucosaminidase and the lack of reaction wi A support this Furthermore the serolog cal reaction with poly C_β shows that these teicho have common antigenic determinants

One difference between poly 2705 and po the degree of sugar substitution The sugar su tion in poly 2705 was found to be only 20 cent whereas 50 per cent of the glycerol resi poly C_β are sugar substituted (2) The effect acetylglucosaminidase on the limited num sugar determinants in poly 2705 elimina serological reaction with serum 3519 but apparent effect on the poly 2705 line which likely due to the glycerol phosphate

Serological differences due to different deg sugar substitution have been demonstrated (2 7) The amount of antibodies with spe against the glycerol phosphate seems to dep the degree of sugar substitution The 2705 rum apparantly contains antibodies primar rected against the glycerol phosphate bu antibody against the sugar

y C_β has earlier (6) been demonstrated gically in several strains belonging to different , of *Staphylococcus* recently described (4 9) resence of the poly C_β precipitation line in 8 f the 10 *S simulans* strains tested is in lance with this β N acetylglucosamine thus to be the immunodominant determinant of ichoic acids of most of these species

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EPIDEMIOLOGY OF *PSEUDOMONAS AERUGINOSA* INFECTION IN PATIENTS TREATED AT A CYSTIC FIBROSIS CENTRE

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Høiby N. & Rosendal K. Epidemiology of *Pseudomonas aeruginosa* infection in patients treated at a cystic fibrosis centre Acta path microbiol scand Sect B 88 125–131 1980

184 isolates of *P aeruginosa* were obtained from the respiratory tract of 45 out of 70 cystic fibrosis (CF) patients at monthly examinations during one year at the Danish CF Centre All isolates were sero-grouped (O antigens) and phage typed and in this way 99 per cent of the isolates could be grouped and/or typed The isolates from CF patients belonged to many different sero groups but 55 per cent were polyagglutinable and most of these belonged to the 0–3/9 complex This was significantly different from ... f ... f

... which was isolated from 10 (22 per cent) of the patients belonged to the 0–3/9 complex and was lysed by phage 109 either alone or in combination with a few other phages Furthermore in a few cases the eradication of another strain of *P aeruginosa* by chemotherapy was followed by colonization of the lungs with the above men... is ... o ... p

... ... of CF patients

Key words *Pseudomonas aeruginosa* epidemiological markers sero-grouping bacteriophage typing cystic fibrosis cross infection

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...ived 6 x 79 Accepted 11 xii 79

Pseudomonas aeruginosa notably mucoid strains ...ur frequently in the respiratory tract of cystic ...osis (CF) patients in contrast to most other ...ups of patients (16) The reason for this ...ciation between CF and *P aeruginosa* is not ...wn but the possibility of cross infection exists ...augment the prevalence of ... ...se bact... ...ures w... ...ients are not kept separate from each other (3, 28)

The aim of the present study was to investigate by means of O grouping and phage typing the epidemiology of *P aeruginosa* infection in the CF patients observed at the Danish CF Centre during one year

MATERIALS AND METHODS

Patients and Bacterial Material

In this work a *P aeruginosa isolate* indicates the descendants of a pure culture of *P aeruginosa* isolated from a single specimen from a patient A *P aeruginosa*

strain indicates several separate isolates from one or more patients of *P aeruginosa* assumed by O grouping and phage typing to be descendants of the same isolate

During the period of this study (January 1st to December 31st 1973) about 90 CF patients were observed at the Danish CF Centre as reported previously (10) These patients are examined as out patients each month or further during hospitalization and bacteriological examination of sputum or laryngeal secretion is carried out as reported previously (10 13) During the one year period of the study 551 isolates of *P aeruginosa* were obtained from 551 sputum or laryngeal secretions from 53 of the CF patients (13) and 484 (88 per cent) of these isolates from 45 patients (85 per cent) were available for the present study

Age and sex distribution of the CF patients 21 females 24 males mean age 9 years range ½–25 years 36 of the CF patients were chronically infected (≧ 6 months continuously) and 9 intermittently (10)

Twenty seven of the patients are still alive (1978) whereas 18 have succumbed From 35 of the patients (78 per cent) *P aeruginosa* was isolated one or more times two years later (1975) and these isolates have also been O grouped and phage typed to see whether the same epidemiological types were still present in the patients All the *P aeruginosa* isolates were stored at room temperature in the dark in meat extract agar slants with sealed corks as reported previously (3 25)

The control material consisted of 501 *P aeruginosa* isolates from blood cultures from 501 patients with bacteraemia from hospitals all over Denmark submitted for typing during 1969–1977

Mucoid non mucoid variation is not dealt with in this paper since this phenomenon has been analysed in several papers from this laboratory (3 11 13 15 16 18)

Typing and Grouping Procedure

All isolates originating from each patient were typed and grouped on the same day One subculture was made on 5 per cent blood agar before the typing procedure

Sero grouping This was performed by slide agglutination with live antigens from an overnight 5 per cent blood agar culture The antisera corresponding to Habs O groups 1–12 (19) have been described previously (24 25) Self agglutinating isolates (SA) agglutinated in saline and non groupable isolates (NG) were not agglutinated by any of the sera When interpreting the results the main rule was that if monoagglutinable isolates were serologically different then they were considered truly different (1) However this rule cannot be strictly adhered to because of the related 0–2/0–5 antigens and the related 0–7/0–8 antigens (19 26) Furthermore many of the *P aeruginosa* isolates from CF patients are polyagglutinable (agglutinated by more than one serum) (1 21) and it has been shown that lysogenization can change a monoagglutinating into a polyagglutinating strain (sero conversion) (19 21) In such cases therefore the main method for distinguishing between different strains is not sero grouping but phage typing

Bacteriophage typing was carried out by th… and typing set of 22 bacteriophages (phages 7 16 31 44 68 73 F7 F8 F10 109 119x 352 … M6 C2 C11 C18 C21 188) obtained from th… Infection Laboratory Colindale Avenue Lon… the isolates were not lysed by the pha… concentration of RTD (routine test dilution) … tions of 100 × RTD were used When the pha… patterns were interpreted isolates were allowed … by as much as two strong reactions with … being regarded as different (1 2) Isolates wh… not be lysed by any of the phages by any of the … were designated NTu

The predominating strain belonged to the … complex 3/9 (see Table 2) and was lysed by p… either alone or in combination with other pha… patterns this strain is referred to as 0–3/9 typ…

Precipitating Antibodies against P aerug… nosa

Sera from all CF patients were examined rou… the presence of precipitating antibodies a… *aeruginosa* by means of crossed immunoelectr… as described previously (11 16) The largest n… precipitating antibodies determined in eac… during the period was used for the calculatio… study

Carbenicillin resistant Isolates

Sensitivity to carbenicillin was determined … during the period of study by means of Neo… (Rosco a/s Tåstrup Denmark) as describe… manufacturer (direct method) Isolates were … to be resistant to carbenicillin if the MIC cal… the regression curve of the manufacturer was … ml The composition of the sensitivity medium … reported previously (20)

Statistical Methods

The fourfold table test and the Mann Wh… were used Level of significance 5 per cent (do… tests) (5)

RESULTS

Sero grouping

About 95 per cent of the 484 isolates co… grouped (Table 1) but more than half of t… polyagglutinable The most prevalent O g… the 0–3/9 which was harboured by more … the patients In 10 (22 per cent) of the patie… was a shift of sero group between 0–3 or … or the 0–3/9 complex (see legend to Tab…

Table 2 shows the prevalence of infectious episodes caused by the different … of *P aeruginosa* in CF patients and in anot… of patients serving as control Polyag… isolates belonging to the 0–3/9 complex pr… ted in CF patients in contrast to the cont… where 0–6 was the …

LE 1 *Distribution into O Groups of 484 P aeruginosa Isolates Cultured from 45 Cystic Fibrosis Patients during one Year*

oup	Number of Isolates		Number of Patients Harbouring the Isolates
	4	(40%)	4
	88 (18%)		11 (24%)
	10		5
	5		2
	73 (15%)		10 (22%)
	4		2
	2		2
	7		2
3	1	(55%)	1
/5	25		6
/6	1		1
/9	207 (43%)		25 (56%)
/9	1		1
/9	1		1
/3/9	5		1
/5/9	3		1
/6/9	10		4
/3/5/9	11		1
aggl (SA)	23	(5%)	8
-Group (NG)	3	(0 5%)	3

See footnote to Table 2

[illegible]jority of the isolates from the control group were [illegible]agglutinable and most of these belonged to the [illegible]tually related 0–2/0–5 groups and the mutually [illegible]ted 0–7/0–8 groups The distribution into O [illegible]ups of a large number of non bacteraemic *P [illegible]uginosa* isolates from non CF patients is similar [illegible] that of bacteraemic isolates presented in this [illegible]er (Rosendal manuscript in preparation)

[illegible]ge Typing

The number and percentage of *P aeruginosa* [illegible]lates lysed by the individual phages used for [illegible]ping *P aeruginosa* are shown in Table 3 Fifty [illegible] per cent of the isolates were lysed by phage 109 [illegible] d 39 [illegible]

[illegible]

[illegible] large 1–14) which [illegible] [illegible]e cor [illegible] [illegible]ound [illegible]on ty[illegible]

Combined O grouping and Phage Typing

When these two procedures were combined only five (1 per cent) of the isolates originating from five CF patients were non groupable *and* non typable When the results of the remaining 479 isolates were analysed 13 different epidemiological strains of *P aeruginosa* were present in more than one CF patient (Table 4) thus indicating that cross infections have probably occurred between these patients These cross infecting strains comprise a total of 48 *per cent of all the 484 P aeruginosa* isolates and the [illegible]

[illegible] *aeruginosa* infection This epidemiological *P aeruginosa* strain was rare in the control material ($<$ 1 per cent)

Nine of the CF patients harboured *P aeruginosa intermittently* during the one year period of the study and three of the chronically infected patients were temporarily free from *P aeruginosa* [illegible]

[illegible] these patients the epidemic 0–3/9 type 109 ± strain

strain indicates several separate isolates from one or more patients of *P aeruginosa* assumed by O grouping and phage typing to be descendants of the same isolate

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The predominating strain belonged t[illegible] the O complex 3/9 (see Table 2) and was lysed by pha[illegible] either alone or in combination with other phages [illegible] patterns this strain is referred to as 0–3/9 type

Precipitating Antibodies against P aeruginosa

Sera from all CF patients were examined rou[illegible] the presence of precipitating antibodies a[illegible] *aeruginosa* by means of crossed immunoelectr[illegible] as described previously (11 16) The largest n[illegible] precipitating antibodies determined in each during the period was used for the calculations [illegible] study

Carbenicillin resistant Isolates

Sensitivity to carbenicillin was determined [illegible] during the period of study by means of Neo[illegible] (Rosco a/s Tåstrup Denmark) as described [illegible] manufacturer (direct method) Isolates were co[illegible] to be resistant to carbenicillin if the MIC calcula[illegible] the regression curve of the manufacturer was ≧ [illegible] ml The composition of the sensitivity medium h[illegible] reported previously (20)

Statistical Methods

The fourfold table test and the Mann Wh[illegible] were used Level of significance 5 per cent (t[illegible] tests) (5)

RESULTS

Sero grouping

About 95 per cent of the 484 isolates could [illegible] grouped (Table 1) but more than half of these [illegible] polyagglutinable The most prevalent O gr[illegible] the 0–3/9 which was harboured by more tha[illegible] the patients In 10 (22 per cent) of the patients [illegible] was a shift of sero group between 0–3 or 0/[illegible] or the 0–3/9 complex (see legend to Table [illegible]

Table 2 shows the prevalence of s[illegible] infectious episodes caused by the different O [illegible] of *P aeruginosa* in CF patients and in another [illegible] of patients serving as control Polyagglu[illegible] isolates belonging to the 0–3/9 complex pred[illegible] ted in CF patients in contrast to the control [illegible] where 0–6 was the predominating O group [illegible]

E 1 *Distribution into O Groups of 484 P* aeruginosa *Isolates Cultured from 45 Cystic Fibrosis Patients during one Year*

oup	Number of Isolates		Number of Patients Harbouring the Isolates
	4		4
	88 (18%)		11 (24%)
	10		5
	5		2
	73 (15%)	(40%)	10 (22%)
	4		2
	2		2
	7		2
	1		1
al	25		6
	1		1
	207 (43%)		25 (56%)
	1		1
	1	(55%)	1
3/9	5		1
5/9	3		1
5/9	10		4
3/5/9	11		1
ggl (SA)	23	(5%)	8
Group (NG)	3	(0 5%)	3

e footnote to Table 2

ority of the isolates from the control group were agglutinable and most of these belonged to the ually related 0–2/0–5 groups and the mutually ted 0–7/0–8 groups The distribution into O ups of a large number of *non-bacteraemic P uginosa* isolates from non-CF patients is similar that of bacteraemic isolates presented in this er (*Rosendal* manuscript *in preparation*)

ge Typing

The number and percentage of *P aeruginosa* lates lysed by the individual phages used for ping *P aeruginosa* are shown in Table 3 Fifty e per cent of the isolates were lysed by phage 109

… per cent) of the 45 CF patients harboured *P uginosa* strains which could be lysed by phage 09 Each isolate was lysed on an average by four ages (range 1–14) which is not different *from* he control group and 190 patterns of lysis were und Only four per cent of the CF isolates were on-typable

Combined O grouping and Phage Typing

When these two procedures were combined only five (1 per cent) of the isolates originating from five CF patients were non groupable *and* non typable When the results of the remaining 479 isolates were *analysed 13 different* epidemiological strains of *P aeruginosa* were present in more than one CF patient (Table 4) thus indicating that cross infections have probably occurred between these patients These cross infecting strains comprise a total of 48 *per cent of all the 484 P aeruginosa* isolates and the most prevalent (104 isolates = 21 *per cent)* was the 0–3/9 type 109± strain which was isolated from 10 (22 per cent) of the CF patients with *P aeruginosa* infection This epidemiological *P aeruginosa* strain was rare in the control material (< 1 per cent)

Nine of the CF patients harboured *P aeruginosa intermittently* during the one year period of the study and three of the chronically infected patients were temporarily free from *P aeruginosa* in

… these patients the epidemic 0–3/9 type 109± strain

TABLE 2 *Percentage of Infections Caused by Different O Groups of* P aeruginosa *Isolated from a Group* [cut off] *Fibrosis Patients Treated in the Danish Cystic Fibrosis Centre and a Group of Non Cystic Fibrosis Pa*[cut off] *Bacteraemia from Hospitals all over Denmark*

O Group	Percentage of Infections Caused in CF Patients	non CF Patients with Bacterae[cut off]
0–1	4 4	11 2
0–2[a]		1 8
0–3	12 1	10 0
0–4	5 5	2 0
0–5[a]	2 2	7 4
0–6	11 0	23 0
0–7[a]		1 2
0–8[a]		0 6
0–9	2 2	5 6
0–10	2 2	9 2
0–11	2 2	8 2
0–12		
0–1/10		0 2
0–2/5[a]	6 6	8 0
0–3/9 complex[b]	39 6	2 8
0–5/6		0 2
0–6/10		0 4
0–7/8[a]		3 8
SA + NG	12 1	4 6
Total Number of Infections	91 0 (100%)	501 (100%)

a) Some authors (19 26) do not consider isolates agglutinable by 0–2 + 0 5 and 0–7 + 0–8 as polyagglut[cut off] the 0–2 and 0–5 antigens as well as the 0–7 and 0–8 are related

b) The 0–3/9 complex includes the related polyagglutinable strains 0–1/3 0–3/6 0–3/9 0–6/9 0–7/9 [cut off] 0–3/5/6 0–3/6/9 0–2/3/5/9 as strains isolated subsequently from individual patients chronically inf[cut off] these strains shifted frequently between some of these serogroups

replaced the original strain in two cases following successful chemotherapy against the original strain

On an average two different strains (range 1–4) judged by combined O grouping and phage typing were isolated from each patient during the one year period However in patients harbouring more than one strain during the year one of the strains predominated and on an average 77 per cent of the isolates (range 50–96 per cent) from each of these patients belonged to the predominating strain

P aeruginosa strains were investigated from 35 of the CF patients two years later (1975) and 27 (77 per cent) still harboured the same epidemiological type whereas eight (23 per cent) harboured another type and three of these had contracted the 0–3/9 type 109 ± strain

Correlation between Antibody Response [cut off] *group of* P aeruginosa

On an average CF patients harbo[cut off] *aeruginosa* of the 0–3/9 complex had a s[cut off] ($p < 0.05$) longer duration of the *P a*[cut off] infection (mean 1 8 years range 1/3–6 ye[cut off] significant ($p < 0.02$) larger number of p[cut off] (mean = 16 2 range 0–51) as compar[cut off] patients harbouring *P aeruginosa* belo[cut off] other O groups (mean 0 8 years ran[cut off] years mean number of precipitins 6 4 [cut off] 21) Furthermore CF patients harbouri[cut off] than one epidemiological *P aeruginos*[cut off] during the one year period of the stud[cut off] significantly larger ($p < 0.05$) number [cut off] tins (mean = 16 5 range 0–51) as c[cut off]

3 484 Pseudomonas aeruginosa *Isolates from Fibrosis Patients Number of Isolates Lysed by h of the Bacteriophages of the Typing Set*

teriophage	Number of Isolates Lysed
7	68
16	37
21	54
24	13
31	114 (24%)
44	131 (27%)
68	176 (36%)
73	21
F7	100 (21%)
F8	108 (22%)
F10	0
109	268 (55%)
119x	89
352	115 (24%)
1214	151 (31%)
M4	36
M6	9
C2	57
C11	114 (24%)
C18	9
C21	36
188	127 (26%)
on Typable (NTu)	20 (4%)

CF patients harbouring only one strain (mean = 8 6 range 0–29)

Correlation between Lethality of the Patients and Epidemiological Types of P aeruginosa

CF patients harbouring polyagglutinable strains had a significantly higher lethality (52 per cent) as compared to patients harbouring only monoagglutinable strains (14 per cent p = 0 05) Furthermore CF patients harbouring *P aeruginosa* strains of the *0–3/9 type 109± complex* had a significantly higher lethality (59 per cent) compared with patients harbouring other strains (22 per cent p = 0 05) These results are based on an observation period of five years

Correlation between Resistance to Carbenicillin and Epidemiological Types of P aeruginosa

Eighty four (42 per cent) of the 198 isolates which were tested against carbenicillin were resistant to that drug However, these carbenicillin resistant isolates were distributed into many different epidemiological types without any remarkable difference from the distribution into types of the remaining carbenicillin sensitive isolates

DISCUSSION

Although the 12 O grouping sera used in the present work do not cover all the O groups of *P*

LE 4 *O Groups and Phage Types of Epidemic* Pseudomonas aeruginosa *Strains Isolated From Two or More Cystic Fibrosis Patients*

roup	Phage Type	Number of Isolates	Number of Patients Harbouring the Isolates
1/9	109±[a]	104	10
[illegible]	68/F7	42	4
3	7/44/68/F8/109/119x/1214/M4/C11/C21/188	29	3
2/5	68	3	3
1	24/44/F8/109/352/1214	2	2
4	68/73/119x/C2/C11	6	2
2/5	1214/188u[b]	7	2
2/5	7/16/21/44/68/F7/109/1214/M6/C2/C21	10	2
-6	31/C11/188	9	2
-6	7/24/68	7	2
-6	119x	2	2
-6	7/16/21/31/44/68/F8/109/119x/1214/M4/C11/C21/188	5	2
-6	109/M4	4	2

109± designates strains lysed by phage 109 either alone or (in short patterns) in combinations with other phages

typed at 100xRTD

aeruginosa (19), 95 per cent of the 484 isolates in the present work could be O-grouped, and the additional phage typing left only 1 per cent of the isolates as non-groupable *and* non-typable, thus *underlining the value of the combined grouping-* and typing-procedure In this connection, it should be stressed that the predominance of a single epidemiological type in the material from CF patients is not due to lack of discrimination of the methods used, since the control material and previously published results from this laboratory showed a completely different distribution of epidemiological types of *P aeruginosa* (25)

In contrast to the control group of other patients, nearly half of the isolates from CF patients in the present material were polyagglutinable This is in accordance with results obtained by other authors dealing with *P aeruginosa* from CF patients and other chronic respiratory diseases (19, 21, 26, 28) It has been questioned by *Pitt & Erdman* (26) whether the antigens responsible for these cross agglutinations are O-antigens, although they are heat-stable However, according to our investigations (12, 14) only the polysaccharides are heat-stable, and they contain the O-antigens so it would seem likely that the polyagglutination is caused by antigenic determinants on the O-antigen-carrying polysaccharides The problems of polyagglutinability concerned almost exclusively the predominant 0–3/9 complex

During continuous infections in the individual patients, subsequent isolates frequently changed polyagglutinability although the phage typing results were identical Since polyagglutinability may be caused by lysogenization and can take place both *in vitro* and *in vivo* in experimental animals (19, 21) our results indicate that lysogenization and subsequent seroconversion actually take place *in vivo* in chronically infected CF patients

Another property of *P aeruginosa* isolated from these patients is the ability to produce mucoid substance (3 13 27) which also seems to be phage-dependent (19 21 22) There is positive correlation between these two independent bacterial properties and the antibody response of the host The complex antibody response to multiple antigens from *P aeruginosa* is most pronounced in patients harbou- [illegible] It is [illegible] ween [illegible] ours [illegible] strains with these two properties (3, 17) It is not likely that selection by means of chemotherapy has played any role, since a combination of carbenicillin and tobramycin is used to treat these infections, and the occurrence of carbenicillin-resistant strains was not correlated to the epidemiological type in accordance with other authors (23) and [illegible] strains were sensitive to tobramycin ([illegible] data) This is in accordance with [illegible] published laboratory experiments (21)

From an epidemiological point of [illegible] present results show a high prevalence of th[e] type 109± strain among the patients a[t] Centre This strain is seldom found [in] hospitals in Denmark, which would in[dicate] cross-infection takes place between the C[F] in the centre, as pointed out previously ([illegible] accumulations of »endemic« strains of *P* [*aerugi*]*nosa* have been reported from other CF-c[entres] 6, 9, 27), but the endemic types were diff[erent from] the 0–3/9 type 109± strain at our centr[e]

The subsequent isolation of several epidemic types from the individual CF pa[tients] with one predominating strain has [been] observed by other authors (4) This is pos[sibly] a reflection of the high rate of cross infecti[on] [among] these patients

The 0–3/9 type 109± strain is respo[nsible for] most of these cross-infections This strain [seems to] be especially virulent for our CF patie[nts] associated with prolonged infection and [illegible] of the lethality in our CF patients as [shown] previously (18) This is not a special feat[ure of the] endemic *P aeruginosa* strain at our CF ce[ntre] other authors (7) have described a simila[r] and prevalent *P aeruginosa* strain of anot[her] their CF centre However, despite of the [illegible] of endemic *P aeruginosa* strains, the pro[gnosis of] CF patients treated in such centres is m[illegible] than that of CF patients treated outside t[illegible] (8) and CF patients treated outside the ce[ntres] also contract *P aeruginosa* infection (*Hø*[*iby*, unpub]lished observations) According to thes[e] therefore it is obviously important to id[entify and] eliminate the routes of cross-infection [illegible] centres and possibly also other places [where] patients are treated in order to dim[inish the] prevalence of *P aeruginosa* infection an[d] further reduce the lethality of CF patient[s]

Miss *Annette Frommelt* and Miss *Anni B*[illegible] thanked for skilful technical assistance

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From an epidemiological point of view present results show a high prevalence of the type 109 ± strain among the patients at the Centre This strain is seldom found in hospitals in Denmark which would indicate cross infection takes place between the CF patients in the centre as pointed out previously (18) accumulations of »endemic« strains of *P aeruginosa* have been reported from other CF-centres (6 9 27) but the endemic types were different from the 0–3/9 type 109 ± strain at our centre

The subsequent isolation of several epidemic types from the individual CF patients with one predominating strain has also been observed by other authors (4) This is possibly a reflection of the high rate of cross infection in these patients

The 0–3/9 type 109 ± strain is responsible for most of these cross infections This strain seems to be especially virulent for our CF patients and is associated with prolonged infection and with ... of the lethality in our CF patients as reported previously (18) This is not a special feature of the endemic *P aeruginosa* strain at our CF centre since other authors (7) have described a similar ... and prevalent *P aeruginosa* strain of another ... their CF centre However despite of the occurrence of endemic *P aeruginosa* strains the prognosis of CF patients treated in such centres is much better than that of CF patients treated outside the centres (8) and CF patients treated outside the centres also contract *P aeruginosa* infection (*Høiby* unpublished observations) According to these results therefore it is obviously important to identify and eliminate the routes of cross infection in the centres and possibly also other places where patients are treated in order to diminish the prevalence of *P aeruginosa* infection and thereby further reduce the lethality of CF patients

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OCCURRENCE OF OCHRATOXIN- AND CITRININ-PRODUCING FUNGI ON DEVELOPING DANISH BARLEY GRAIN

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Lillehoj E B & Goransson B Occurrence of ochratoxin and citrinin producing fungi on developing Danish barley grain Acta path microbiol scand Sect B 88 133–137 1980

The distribution of *Aspergillus* and *Penicillium* spp isolates that produce ochratoxin A (OA) and citrinin was studied on developing barley from diverse locations in Denmark during the 1977 crop year From 33 test fields 243 isolates of the two genera were obtained with significant numbers of isolates on the grain at the earliest sampling date (1–2 weeks postanthesis) No specific agronomic factor was linked to the distribution of *Aspergillus* and *Penicillium* spp A taxonomic study of 108 representative isolates of the two genera showed that *A flavus P chrysogenum* and *P purpurrescens* were the predominant species An investigation of the toxin production capability of the identified species showed that seven strains (6 5%) produced OA and a single isolate synthesized citrinin *P purpurrescens* was the dominant OA producing species with four toxin positive isolates

Key words Ochratoxin A citrinin *Aspergillus Penicillium* barley

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…chratoxin A (OA) and citrinin are toxic …tabolites of several *Aspergillus* and *Penicillium* … (7 9) Although the toxins have been detected … a number of commodities at geographically …erse locations the principal interest has involved …dies of cereal feed grains in Scandinavia and food …certain Balkan countries (7 9 14) OA is a major …ease determination of porcine nephropathy (9) a …urally-occurring disease in Denmark (7 8) and …eden (14) Presence of OA in feed samples …marily barley has been linked to increased …currence of the swine disorder (7 9)

A kidney disease of humans in certain areas of …lgaria Rumania and Yugoslavia has been related … ingestion of OA (9) A study of foods in these …gions has identified a high incidence of the toxin …3) In addition an association between the kidney …sease and enhanced rate of urinary tract tumors … n reported (1)

The fungi that produce OA and citrinin are widely distributed and have been identified in many foods and feeds (8 9) The microbes that produce the toxins have traditionally been characterized as »storage« or »postharvest« fungi because their ecological niche is determined by water activities routinely observed in mature grain (11) Since recent studies have shown that storage fungi can infect immature maize kernels in the field, the delineation between storage and field fungi is ambiguous (12) A particularly perplexing problem in these investigations of mycotoxin contaminations has been the question of the origin of the initial fungal inoculum Previous investigations of the mycoflora of developing and freshly-harvested barley have demonstrated a limited occurrence of *Aspergillus* and *Penicillium* spp, but the OA- or citrinin production potential of the isolates was not identified (4, 5, 6)

1 Distribution of Aspergillus and Penicillium spp Obtained from Danish Barley after Incubation at 10° and 28 °C

tion rature)	Grain Treatment	Number of Fungal Isolates[a]						Totals
		Aspergillus Sample Date			*Penicillium* Sample Date			
		1	2	3	1	2	3	
10	A	1	–	–	17	–	5	23
10	K	1	–	2	27	8	21	59
28	A	9	14	20	16	6	9	74
28	K	16	17	16	21	9	8	87
		27	31	38	81	23	43	243

mber of *Aspergillus* and *Penicillium* isolates obtained from wash of 100 grains per sample date per field and ing 36 grains samples per sample date per field Two Petri plates of aqueous suspension (A) and 3 of barley nels (K) were incubated at each temperature of material from each field

tes obtained from all of the fields suggested age incidence of about 3 isolates per field riate consideration of aliquot volumes and is demonstrated that a total of 3750 *Asper* nd *Penicillium* isolates were present in the wash of 300 grains from each field The 146 isolates from all washed kernels (K) related rage distribution of 4 4 isolates/field based s value the 108 kernels plated per field ed an occurrence of 3 2% of *Aspergillus* and *lium* isolates

resentative colonies (108) of the 243 *Asper* and *Penicillium* spp were characterized omically (Table 2) The isolates chosen for s identification were selected because of the ncy of occurrence of similar colonies (gross hology and color) Of the 21 *Aspergillus* s 14 were *a flavus* none of the isolates ced aflatoxin The predominant *Penicillium* *cyclopium* comprised about one third of the s isolates *P purpurrescens* and *P chrysogenum* also common The three major *Penicillium* es provided approximately 60% of all the tified isolates Tests for production of OA and nin by each of the characterized species on le barley demonstrated that six isolates produ OA at 28 °C and two at 10 °C with only a le citrinin producing isolate (28 °C) The single *chraceus* isolate obtained in the study produced at both production temperatures the remaining -producing species were an unidentified *Penicil m sp* *P purpurrescens* and *P verruculosum* An late of *P granulatum* produced citrinin

Examination of the origin of the toxin producing species showed that seven of the eight were obtained from initial incubations of 28 °C with only a single isolate from 10 °C (Table 3) Four of the isolates were obtained from initial aqueous suspensions and four from washed barley grain An isolate of *P purpurrescens* obtained from 10 °C incubation produced OA only at 28 °C and another isolate of the same species obtained from 28 °C incubation produced OA exclusively at 10 °C The remaining isolates were all obtained from 28 °C incubations and produced toxin at 28 °C The seven OA-producing species found on Danish barley represented a 6 5% incidence of the 108 isolates taxonomically identified and a 2 9% occurrence of all of the *Aspergillus* and *Penicillium* spp obtained in the study Although no definite association was observed between the origin of toxin producing species and agronomic practice toxin producers were obtained from fields with a below average incidence of total *Aspergillus* and *Penicillium* isolates in six of the eight instances

DISCUSSION

Although the preponderant mycoflora on developing barley grain consisted of «field» fungi (*Alternaria Cladosporium Cephalosporium Fusarium* spp) a significant incidence of *Aspergillus* and *Penicillium* spp was present on grain at all stages of development Of the two preponderant *Penicillium* spp *P cyclopium* and *P purpurrescens* only

The current study was undertaken to 1) provide information on the presence of OA- or citrinin producing fungi in developing Danish barley grains 2) elucidate interregional variation in the incidence of the fungi and 3) study agronomic factors that might contribute to selection of toxin producing fungal isolates

MATERIALS AND METHODS[a]

Thirty three fields of Danish barley were selected for sampling in 1977 from diverse locations including North Zealand 4 South Zealand 8 Falster 2 Bornholm 2 Funen 2 North Jutland 10 and South Jutland 5 Agronomic information acquired from each test field included barley variety dates of planting and anthesis and pesticicde application Samples were collected at three stages of grain maturity 1) 1–2 weeks after anthesis 2) 3–4 weeks after anthesis and 3) physiologically mature seed Three representative locations (10–20 m²) were selected for sampling within each field and 10 heads were collected per location Sample heads from each field were securely wrapped in water repellant bags and transported to Copenhagen for processing In the laboratory grains were carefully removed from heads to avoid inadvertent fungal contamination A 100 grain sample that was representative of the 30 heads from a field was placed in a sterile beaker and immersed in 25 ml of a sterile Triton X (0 01%) solution The suspension was gently shaken for one min with subsequent dilution (1/10) transfer and spreading of a 0 2 ml aliquot on Petri plates containing ME agar (2% malt extract 2% agar pH 4) (3) The diluted suspension provided a microbial population that allowed for effective discrimination of *Aspergillus* and *Penicillium* spp

Test grains were subsequently washed five times with 50 ml of sterile Triton X Aliquots from the last wash yielded less than five colonies/plate Six washed barley grains were transferred to each test plate (2% ME) by sterile procedure Four plates of each initial wash suspension and six plates of washed grain were prepared from material obtained from each test field one half of the plate were incubated at 28 °C for 10 days and the remainder at 10 °C for three weeks The 10 °C incubation was employed because *Scott et al* (15) had observed that low temperatures permitted outgrowth of *Penicillium* spp that failed to compete at higher temperatures

... and ... of ...

isolates obtained form 28 °C incubations were incubated at 28 °C for 10 days and isolates from 10 °C were subsequently incubated at 10 °C for 3 weeks ... isolates obtained from Czapek plates were ... and maintained on Czapek agar slants

Testing individual *Aspergillus* and *Pen*... OA citrinin and aflatoxin production was ... culturing the fungi on pearled barley ... water and 50 g barley (300 ml Erlenmeyer) ... for 12 h before autoclave sterilization ... 121 °C Duplicate flasks of each test were ... with 1 ml suspensions of spores and sets were ... at 28 °C for 10 days and 10 °C for 3 week ... were extra...

reduced in volume by evaporation ... solutions were spotted on thin layer chrom... (TLC) plates coated with 0 5 mm of Adsorb... plied Scienced Laboratories State College Pa ... ping solvents included 1) benzene acetic acid ... v) 2) benzene methanol acetic acid (90 5 5 ... hexane acetone acetic acid (90 10 5 v/v) and ... ene ethyl acetate formic acid (60 30 10 v/v) P... mycotoxins was determined by equivalent m... test spots with reference standards of pure t... was verified by formation of the ethyl ester (...

RESULTS

During the 1977 crop year 243 *Asper*... *Penicillium* isolates were obtained from ... fields of Danish barley The average nu... isolates per field was 7 4 but large vari... observed with numbers isolated from spe... ranging from 0–22 Barley kernels fro... fields yielded some *Aspergillus* or *P*... isolates The data indicated a definite hete... between fields in numbers of the fungal sp... no distinct interregional differences The ... appearance of the fungi was examined by ... the barley grain at three maturity stages T... demonstrated an early presence of the *A*... and *Penicillium* spp but no significant d... between sampling periods Although a ... higher incidence of *Penicillium* spp than *A*... isolates was observed in material from ... sampling period less variation in numbe... two genera was found at later sampling t...

A comparison of the origin of the fung... from either the initial aqueous suspensio... washed kernels (K) demonstrated that mor... were obtained from kernels than from ... suspensions (Table 1) More *Aspergillus* ... were obtained from the 28 °C incubation th... 10 °C incubations but *Penicillium* isola... often more frequent after incubation at 10 ... 300 grains (100 × 3 sampling times) wer... from each field for the initial wash ...

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[a] Names of companies or commercial products are given solely for the purpose of providing specific information their mention does not imply recommendation or endorsement by the U S Department of Agriculture over others not mentioned

as a synergist in mediating the kidney
3 9)

rs wish to thank *D T Wicklow* NRRC oria Ill for identification of *Aspergillus* and spp *A Jensen B Welling* and *J Reitzel* irch Center for Plant Pathology Lyngby for in acquisition of samples and valuable ; and the Department of Plant Pathology and Agric Univ for in providing laboratory The research was supported by a grant from *Agricultural and Veterinary Research Council* 1059)

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TABLE 2 *Distribution of Ochratoxin and Citrinin-Producing Fungal Species Obtained from Danish Barley*

Aspergillus and *Penicillium* spp	Total	Number: Ochratoxin Producers 10 °C	Number: Ochratoxin Producers 28 °C	Number: Citrinin Producers 10 °C	Number: Citrinin Producers 28 °C
A ficum	1	–	–	–	–
A flavus	14	–	–	–	–
A fumigatus	4	–	–	–	–
A ochraceus	1	1	1	–	–
A repens	1	–	–	–	–
Penicillium spp	3	–	1	–	–
P charlesii	1	–	–	–	–
P chrysogenum	15	–	–	–	–
P corylophilum	2	–	–	–	–
P cyclopium	28	–	–	–	–
P funiculosum series	1	–	–	–	–
P granulatum	2	–	–	–	1
P nigricans series	1	–	–	–	–
P purpurrescens	22	1	3	–	–
P raistrickii	1	–	–	–	–
P roqueforti	5	–	–	–	–
P stoloniferum	4	–	–	–	–
P thomii	1	–	–	–	–
P verruculosum	1	–	1	–	–
Total	108	2	6	–	1

[a] Number of isolates producing OA or citrinin on sterile barley at 28 °C for 10 days and 10 °C for three weeks

isolates of the latter species produced O[...] though other studies have shown that both [...] produce the toxin No *P viridicatum* stra[...] isolated from developing barley grain. No [...] agronomic factors or interregional differenc[...] be linked to the occurrence of OA or [...] producing fungi

The predominance of *P purpurrescens* [...] OA producers isolated from the Danish ba[...] somewhat unexpected because earlier r[...] toxin production by the species had I[...] with isolates obtained from meat (2) The [...] in the toxin-production character in the [...] nant species and the total absence of OA [...] in *P cyclopium* isolates raises a questi[...] factors in the microbial milieu that c[...] character for toxin production

The relatively common occurrence of [...] and *P cyclopium* observed in the curr[...] corroborate observations made by *Wellin*[...] study of Danish barley in 1970 Th[...] investigation had also routinely identified [...] *losum* and *P hordei*, isolates of these sp[...] not dominant microbes in the 1977 [...] results suggest that environmental va[...] tween crop years may exert a selective [...] closely related microbes that occur on [...] barley

Production of citrinin by *P granulatu*[...] in this study increase the number of fu[...] that have been reported to synthesize th[...] However, the paucity of citrinin produc[...] from Danish barley corroborates earli[...] tions of the limited importance of ci[...] decisive disease determinant in mycoto[...] nephropathy although the metabolite [...]

TABLE 3 Origin of Fungal Isolates that Produced Ochratoxin A or Citrinin

Fungal Isolate	Isolation[a] Temp (C)	Production Temp (C)[b]: OA	Production Temp (C)[b]: Citrinin	Fie[...]: Ident No	Fie[...]: A[...] and [...] s[...]
P purpurrescens	10	28	–	1	
P purpurrescens	28	10	–	11	
A ochraceus	28	10 28	–	11	
P purpurrescens	28	28	–	12	
P purpurrescens	28	28	–	18	
Penicillium sp	28	28	–	19	
P granulatum	28	–	28	21	
P verruculosum	28	28	–	24	

a
b [...] 28 °C for 10 days or 10 °C for 3 weeks

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EVALUATION OF ELISA IN THE DIAGNOSIS OF *YERSINIA ENTEROCOLITICA* DIARRHOEA IN CHILDREN

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Vesikari T Granfors K Maki M & Gronroos P Evaluation of ELISA in the diagnosis of *Yersinia enterocolitica* diarrhoea in children Acta path microbiol scand Sect B 88 139–142 1980

In a series of acute gastroenteritis in children including 2 bacteriologically confirmed cases of *Yersinia enterocolitica* infection paired sera from 286 patients were studied for the presence of *Y enterocolitica* 0 3 and 0 9 antibodies using an enzyme linked immunosorbent assay (ELISA) In the 2 cases with positive stool culture of *Y enterocolitica* 0 3 a significant rise was seen in each IgM IgG and IgA class *Y enterocolitica* 0 3 antibodies There were 6 further cases with elevated anti Yersinia IgM these possibly false positive reactions were distinguished from the confirmed cases of *Y enterocolitica* infection by the absence of IgG and IgA antibodies

Key words ELISA *Yersinia enterocolitica* diarrhoea gastroenteritis

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Enzyme linked immunosorbent assay (ELISA) *Yersinia enterocolitica* antibodies of different immunoglobulin classes (2 3) has appeared useful the retrospective diagnosis of Yersinia arthritis distinctive antibody pattern with prolonged persistence of Yersinia-specific IgG and IgA antibodies is often present in such patients (4)

In children the most common form of yersiniosis an acute abdominal disease with or without diarrhoea (7) At the acute stage the diagnosis of Yersinia may be more readily based on bacterial culture but also in these cases the diagnosis may be missed because of inadequate specimen collection or bacteriological techniques The present study was undertaken to establish whether the newly-developed ELISA which detects IgM IgG and IgA-class antibody responses would reveal more cases of Yersinia infection than bacteriological studies in a series of gastroenteritis in children

MATERIALS AND METHODS

Sera The serum specimens for ELISA were derived from a prospective study of gastroenteritis in children aged 0 to 15 years lasting from December 1 1977 to November 30 1978 Paired sera were available from 286 patients with acute diarrhoea admitted to the Department of Paediatrics Tampere Central Hospital The serum specimens were collected from each upon admission and at convalescent stage 3 to 4 weeks later

The sera were studied retrospectively under code for *Yersinia enterocolitica* 0 3 and 0 9 IgM IgG and IgA class antibodies using ELISA (see below)

Microbiological studies From each patient 1 to 3 stool specimens were taken for bacterial and viral studies At each time of stool specimen collection a microscopic

[illegible]

[illegible] enteric pathogens were searched for Campylobacter Salmonella Shigella invasive *E coli* toxigenic (LT) *E coli* and pathogenic serotypes of *E coli*

ersinia enterocolitica 0 3 antibodies In the ise the IgM antibody response was also strong stinctive, whereas in the second case the IgM dies did not increase much The results were ially similar using the LPS antigen

iddition to the two bacteriologically confirmed there were 6 further patients with negative iological studies but with ELISA findings ily suggesting recent infection with *Y entero-a* (Table 2) In each case there was some ise of anti-Yersinia IgM antibodies measured iole bacteria antigen and/or the concentration it Yersinia IgM in the second serum specimen it least 21%

ith regard to the IgG and IgA-class anti nia antibodies the bacteriologically negative were clearly different from the two confirmed of yersiniosis The IgG and IgA antibody ntrations were low and there was little or no ase in them by the convalescent stage (Table 2) n the LPS antigen was used for ELISA the values exceeded 21% in 5 out of 6 cases No ases were seen in the IgG and IgA class anti inia antibodies using the LPS antigen or *Y rocolitica* 0 3 heat treated antigen

linically the two bacteriologically proven cases ersiniosis had exudative diarrhoea with faecal ocytes This is consistent with an enteroinvasive iogen such as *Y enterocolitica* (8) Among the aining suspected cases there were 2 with lative and 4 with nonexudative diarrhoea Other robiologic findings in these patients are shown Table 2

DISCUSSION

conclude that serological studies with ELISA not reveal any additional cases of yersiniosis in present series of childhood diarrhoea Those h positive bacterial cultures also had a distinctive Yersinia antibody response in all immunoglo-in classes In addition among the bacteriologi ly negative patients there were a few cases with ne increase in anti Yersinia IgM antibodies but th no concomitant rise in IgG or IgA antibodies The present findings may provide guidelines for interpretation of Yersinia serology involving ferent immunoglobulin classes Until larger aterials of acute yersiniosis are studied it seems fe to suggest that for a positive diagnosis of acute *enterocolitica* infection a rise should be detected each IgM IgG and IgA anti Yersinia antibodies lso diagnosis of recent Yersinia infection based on e demonstration of anti Yersinia IgM antibodies a single serum specimen must be viewed critically in the absence of simultaneous high levels of Yersinia antibodies of other immunoglobulin classes

The origin and nature of the »nonspecific« anti-Yersinia IgM antibody increases in the bacteriologically negative cases remains unresolved The well-known cross reaction between *Y enterocolitica* and *Brucella abortus* (1) is excluded since this cross-reaction can be avoided by the use of purified LPS antigen (3, 6) It is also unlikely that the observed high titers of *Mycoplasma pneumoniae* CF (Table 2) antibodies in these patients result from true Mycoplasma infections *Instead we suggest that* both the apparent Yersinia IgM- and Mycoplasma antibodies represent cross reactions with unknown microbial or other antigens or that they result from general humoral immune stimulation associated with gastrointestinal infection (5)

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TABLE 1 Yersinia enterocolitica *0 3 IgM- IgG- and IgA class Antibodies Measured by ELISA in Two Ch[illegible]* *Bacteriologically Confirmed* Y enterocolitica *0 3 Infection*

Patient	Age	Anti Yersinia 0 3 antibody concentration per cent of standard					
		IgM		IgG		IgA	
		I[a)]	II[b)]	I	II	I	II
1	5 yr 9 mo	7	288	7	>500	4	[illegible]
2	3 yr 8 mo	4	21	2	105	6	[illegible]

a) I = acute stage
b) II = convalescent stage (4 weeks)

rotavirus and adenovirus These results have been reported in part previously (9)

ELISA procedure The ELISA method has been described in detail previously (2 3) In brief the assay was a double antibody method utilizing immunoglobulin class specific rabbit anti human sera and swine anti rabbit IgG alkaline phosphatase conjugate The antigen preparations used were formalized whole *Y enterocolitica* 0 3 bacteria (11) heat treated (100 °C for 1 h) *Y enterocolitica* 0 9 bacteria and lipopolysaccharide (LPS) from *Y enterocolitica* 0 3 (6) All ELISA determinations were made in duplicate The mean absorbance values of the two determinations were compared to standard sera separate for IgM IgG and IgA which had been termed to have a 100% concentration of the corresponding antibody (2) Accordingly the results of ELIS[illegible] expressed as per cent values The cut-off po[illegible] positive reaction for IgM was 21% and for IgG [illegible] 7% and 8% respectively These limiting val[illegible] obtained by testing negative sera described pre[illegible] (2)

RESULTS

Y enterocolitica 0 3 was isolated from 2 ca[illegible] acute diarrhoea in the series The corresp[illegible] anti Yersinia 0 3 antibody concentrations me[illegible] by ELISA are shown in Table 1 In both case[illegible] was a very clear increase of both IgG and Ig[illegible]

TABLE 2 Yersinia enterocolitica *0 3 IgM IgG and IgA class Antibody Concentrations Measured by ELI[illegible]* *Patients with Acute Diarrhoea Negative Bacterial Cultures for* Y enterocolitica *and Possible False Positive* [illegible] *Serology*

Patient	Age	Anti Yersinia enterocolitica 0 3 antibody concentration per cent of standard						Other microbiolo[illegible] findings
		IgM		IgG		IgM		
		I[a)]	II[b)]	I	II	I	II	
3	1 yr 2 mo	39	21	28	9	9	9	rotavirus +
4	3 yr 4 mo	30	39	10	8	19	23	mycoplasma CF I 4096 II 4096
5	3 yr 11 mo	7	22	6	16	4	2	mycoplasma CF I 32 II 256
6	8 yr 2 mo	8	90	3	16	18	25	mycoplasma CF I 4 II 256
7	8 yr 4 mo	13	24	9	10	9	13	invasive *E coli*
8	11 yr 4 mo	28	42	13	18	14	21	–

a) I – acute stage
b) II = convalescent stage (4 weeks)

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AN ANTIGEN COMMON TO A WIDE RANGE OF BACTERIA

I The Isolation of a Common Antigen from Pseudomonas aeruginosa

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Sompolinsky D Hertz J B Høiby N Jensen K Mansa B & Samra Z An antigen common to a wide range of bacteria I The isolation of a Common Antigen from *Pseudomonas aeruginosa* Acta path microbiol scand Sect B 88 143–149 1980

In crude water soluble extracts of *Pseudomonas aeruginosa* 64 antigens can be demonstrated by crossed immunoelectrophoresis in agarose with polyvalent Pseudomonas immunoglobulin One of these antigens cross reacts with antigens prepared from bacteria of a wide range of taxonomic groups Monospecific immunoglobulins to this antigen (Common Antigen) were produced by immunization with the appropriate immunocomplex extracted from agarose Common Antigen was purified by the combination of two fractionation methods Precipitation of the crude extract with 18% (w/v) sodium sulfate followed by gel filtration of the supernatant on a Sephadex G 200 column By this method 35% of Common Antigen from the crude extract was recovered more than half of the fractions electrophoretically pure Electrophoresis of reduced Common Antigen on a dodecyl sodium sulfate polyacrylamide gel revealed two protein bands with apparent molecular weights of 59–62000 and 62–65000 respectively The untreated antigen however passed a column of Sephadex G 200 with the void volumen indicating a substance of high molecular weight (> 4 600000)

Key words *Pseudomonas aeruginosa* Common Antigen isolation Na_2SO_4 precipitation gel filtration crossed immunoelectrophoresis

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Many investigators have in recent years shown interest in the occurrence of serological cross reactions between different bacteria Cross reacting antigens may be of importance as immunogenic agents by inducing protection against infections with remote pathogenic microorganisms (18) Furthermore if a cross reacting antigen is shared by an entire taxon of higher or lower range it may by part of a vital biochemical structure that has been preserved through phylogenesis A representative of the latter kind of cross reacting antigens is the substance designated OEP of *Pseudomonas aeruginosa* (13) This substance is part of the endotoxin Another representative is the Enterobacterial Common Antigen which is a part of the cell wall of *Enterobacteriaceae* (16)

In an investigation of immunological cross reactions between *Pseudomonas aeruginosa* and 36 other bacterial species *Høiby* reported (7) that 30 of them representing a wide range of gram negative and two gram positive microorganisms showed cross reactions with the antigen represented by

[1] On leave from Department of Microbiology Assaf Harofe Government Hospital Zerifin and Department of Biology Bar Ilan University Ramat Gan Israel

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After removing the polyacrylamide strip the ...e plate was washed dried stained and destained as ...sed previously (11)

...n Determination

...tein content of antigen solutions was determined ...ding to the method of *Lowry et al* (15)

...icals

...phadex G 200 Sephadex A25 DEAE and a ...ure of proteins of known molecular weight (LMW ...

...se (Indubiose A 37) was purchased from ...ustrie Biologique Francaise France Acrylamide ... Bio Rad Laboratories Richmond Calif N N ...

RESULTS

... CA

from agarose seemed in crossed immunoelec...oresis with St Ag to be monospecific for CA ...1B)

...ion of CA by Salting out and Sephadex gel ...ation

... many of the fractionation techniques applied ...n-exchange chromatography acid precipita... ammonium sulfate and sodium phosphate ...pitation) fractions with a high concentration of ... were obtained and only 5–15 other antigens ... demonstrated by Ciwig By combining two or ...e of these techniques a higher degree of purity ...ld be obtained It was however remarkable that ... occurred in the same fractions as antigen 37 a ...saccharide-containing antigen giving a heavy ...cipitate on Ciwig with St Ab in the second ...ension gel (9) By gel filtration on Sephadex ...200 CA and antigen 37 were also recovered ...ether in fractions corresponding to the void ...umen of the column However 18 % Na_2SO_4 ...ved very efficient in precipitating antigen 37 ...hile leaving the majority of CA in the supernatant ...able 1) together with about 25 other antigens ...hen this Na_2SO_4 Ag was applied to columns of ...200 CA could be demonstrated by rocket ...munoelectrophoresis in a few of the fractions ...ly The elution volumen for peak concentration of ...A corresponded to that of Blue Dextran of a mean ...ol wt of 2×10^6 i.e. CA appeared in the void

A

StAg/NaCl/StAb

B

HSA

StAg/MonospAb/StAb

Fig 1 Analysis of the purified and concentrated solution of immunoglobulins G and A to Common Antigen (anti CA) by crossed immunoelectrophoresis with intermediate gel The antigen was 2 µl of the polyspecific Standard Antigen throughout

A Reference system with 20 µl/cm² of the polyspecific Standard Antibody in the second dimension gel and no antigen or antibody in the intermediate gel Common Antigen is indicated by an arrow

B As A but with 200 µl anti CA in the intermediate gel The mobility of human serum albumin (HSA) is indicated Staining Coomassie Brilliant Blue R Anode to the right (first dimension) and to the top (second dimension)

volumen In a typical experiment 147 5 mg antigen protein in 10 ml buffer was applied to a 120 × 2 5 cm column of Sephadex G 200 By fused rocket electrophoresis with anti-CA in the intermediate gel CA was demonstrated in 17 fractions of 4 ml

precipitate No 10 of the crossed immunoelectrophoretic reference system for *P aeruginosa* (8) This antigen will in the present communication be designated Common Antigen (CA) The purpose of this paper is to describe the techniques used to isolate CA from *P aeruginosa* and the purity and yield of the product achieved

MATERIALS AND METHODS

Reference Antigen and Antibody

The *P aeruginosa* reference system (St Ag/St Ab) which shows at least 64 different immunoprecipitates in crossed immunoelectrophoresis with intermediate gel (Ciwig) was used as previously described (8 10)

Preparation of Rabbit Immunoglobulins Specific to CA

After Ciwig with St Ag against an antiserum to *Klebsiella pneumoniae* (7) the precipitate of CA (precipitate No 10 see ref 7) can be recognized even without staining An appropriate part of the agarose containing this precipitate and no other visible precipitates was cut out (4 20) and washed with isotonic saline for 24 h suspended in dest water and sonicated for 30 seconds (20 000 cps ice cooled) (9) The sonicate was mixed with an equal volume of Freund s incomplete adjuvant and stored at −20 °C until use Immunization of three rabbits was performed according to *Harboe & Ingild* (5) Each rabbit received 100 µl immunogen per immunization and the rabbits were immunized for a period of 12 months A solution of immunoglobulins A and G (anti CA) was prepared as described for St Ab (11)

Antigen for the Isolation of CA

A crude extract of *P aeruginosa* 888 (O group 5 one of the 4 strains of St Ag) was obtained with a French Press and used for the isolation of CA Thirty grams wet weight of the bacteria suspended in 40 ml dist water was passed once through the press at 10 000 p s i After disintegration the extract was incubated for 30 min at 4 °C with deoxyribonuclease and magnesium acetate at final concentrations of 10 µg/ml and 45 mM respectively (pH 7 4) The remaining whole bacteria and coarse material were separated by centrifugation at 5 000 × g for 15 min and cell walls and cell membranes were sedimented at 48 000 × g for 30 min The supernatant was passed through a 0 22 µm Millipore® filter

Fractionation of Pseudomonas Extract

The crude water soluble extract prepared from *P aeruginosa* 888 was fractionated by a number of conventional techniques including salting out with various concentrations of sodium phosphate sodium sulfate and ammonium sulfate acid precipitation at pH 4 0 and 4 8 anion exchange chromatography with a chessboard gradient of NaCl and hydrogen ion concentrations and gel filtration on columns of Sephadex G 200 The fractions were analysed for the presence of CA and other antigens by rocket immunoelectrophoresis (21) fused rocket immunoelectrophoresis with intermediate gel (1) and Ciwig with St Ab and anti-CA result of this analysis the following method wa[s] as a standard procedure for the isolation of [CA]

a To the crude bacterial extract was add[ed] anhydrous Na_2SO_4 per ml The powder soon a compact white flake that was dissolved magnetic stirring (about 2 h at room tp) M[...] heavy turbidity of precipitated material app[...] this was separated from the supernatant by [...]tion The supernatant was dialysed against tw[o] of 200 volumes of 40 mM trisbuffer pH dialysed solution will in the following be Na_2SO_4 Ag In some cases the sediment [...] precipitation was dissolved in 40 mM trisbuff[er] as above and studied separately

b Samples of Na_2SO_4 Ag were applied to [a] Sephadex G 200 which had been equilibra[ted] solution of 0 15 M NaCl 10 mM tris 0 1% 7 2 The fractions collected were examined electrophoresis fused rocket electrophoresis [with inter]mediate gel and Ciwig The void volume was [...] with Blue Dextran (mean MW 2 × 10⁶) S[...] fractions from the G 200 column were furthe[r] on a column of anion exchange gel (Sepha[...] DEAE) equilibrated with sodium phosphate [...] mM pH 6 0) The antigen was eluted by a c[...] gradient of NaCl concentration (0–0 8M) in buffer

Polyacrylamide electrophoresis with Dodecyl Sulfate (SDS PAGE)

SDS PAGE was performed on vertical 8 × [...] cm slabs at 200–250 mV till the bromphenolbl[ue] reached the lower edge of the slab The gels polyacrylamide and 0 19% N N methylene[bisacryla]mide were formed in a buffer of 200 mM phosphate with 0 2% SDS and 6M urea (pH [...] electrophoresis buffer contained 100 mM phosp[hate] mM Na+ 5 mM Cl and 0 1% SDS (pH [...] samples were diluted 1 3 and heated to 60 °C [...] the following buffer 10 mM phosphate 2 5 1 5% dithiothreitol 9M urea pH 7 0 After ele[ctropho]resis the slabs were stained with Coomassie Blue R (0 1 % in 0 28 M trichloracetic acid and sulfosalicylic acid) during 1 h at 57 °C and 57 °C in two portions of trichloracetic acid (0 65 kit of low molecular weight proteins (Pharmac[ia] used for calibration of the linear best fit rela[tion] between R_f and log of MW (3 19)

Crossed SDS PAGE Agarose Immunoelectrophoresis (17)

Sample trails of SDS PAGE were washed 20 room temperature in the tris barbital buffer [...] used for immunoelectrophoresis Then the str[ips] placed on a 2 5 cm wide band of 1 5 % agarose [...] usual tris barbital buffer (pH − 8 6 Γ/2 = 0 [...] 5 % Triton X 100 on a 7 × 7 cm glass plate The dimension gel contained 1 % agarose 1 % Triton and 2 µl/cm² anti CA The proteins of the po[lyacryla]mide gel were run into the [...]

fter removing the polyacrylamide strip the late was washed dried stained and destained as previously (11)

eterminat on

n content of antigen solutions was determined g to the method of *Lowry et al* (15)

ls

dex G 200 Sephadex A25 DEAE and a of proteins of known molecular weight (LMW obtained from Pharmacia Fine Chemicals AB Sweden Urea glycine dodecyl sodium sulfate sie Brilliant Blue R and Blue dextran 2 000 were d from Sigma Chemical Co St Louis Miss (Indubiose A 37) was purchased from trie Biologique Francaise France Acrylamide o Rad Laboratories Richmond Calif N N ne bisacrylamide and sodium sulfate from BDH als Ltd Poole England and Triton X 100 from Berntsen AS Copenhagen

RESULTS

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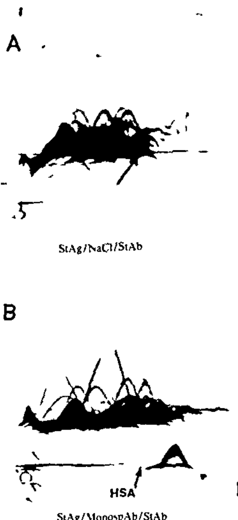


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A Reference system with 20 µl/cm² of the polyspeci fic Standard Antibody in the second dimension gel and no antigen or antibody in the intermediate gel Common Antigen is indicated by an arrow

B As A but with 200 µl anti CA in the intermediate gel The mobility of human serum albumin (HSA) is indicated Staining Coomassie Brilliant Blue R Anode to the right (first dimension) and to the top (second dimension)

volumen In a typical experiment 147 5 mg antigen protein in 10 ml buffer was applied to a 120 × 2 5 cm column of *Sephadex* G 200 By fused rocket electrophoresis with anti-CA in the intermediate gel CA was demonstrated in 17 fractions of 4 ml

TABLE 1 *Isolation of Common Antigen Concentration of Protein and Common Antigen in Various Frac...*

	Vol in ml	Protein (mg/ml)	Concentration in % of crude extract	Common Antigen Arbitrary Units per ml	per mg protein	... (recov...)
Crude French Press extract of *Pseudomonas aeruginosa*	10	14 7	–	100	6 80	100
Na_2SO_4 Ag[1]	10	4 75	32 3	50	10 53	50
Pooled fractions from Sephadex G 200 chromatography[2,3]	68	0 055	0 37	5 1	92 7	...
Fraction 24 from Sephadex G 200 chromatography	4	0 126	0 86	13 5	107 14	

1) Supernatant after precipitation of crude extract with 18 % Na_2SO_4
2) The examinations were performed with a mixture of equal volumes of the 17 fractions that contained CA
3) See Fig 3

each (Fig 2) Ciwig with individual fractions of antigen in the first dimension and with St Ab in the second dimension gel indicated that 9 of the 17 fractions including those with peak concentration of CA were immunoelectrophoretically pure and in the other 8 fractions only one additional precipitating antigen was demonstrated When a pool of all 17 fractions was applied to a column of A25 DEAE and eluted with a gradient of NaC... phosphate buffer CA appeared in the fractions... 0 3–0 4 M NaCl and no other antigen demonstrated in these fractions

Apparently pure fractions obtained by G... filtration were pooled and concentrated 5 times... an Amicon B15 concentration set (Amicon C... Lexington Mass) and 10 µl of the concentrate... examined on SDS PAGE Two bands with close R_f-values were revealed the heavier fainter than the lighter one R_f-values for t... bands were somewhat dependent on the ...ture used for denaturation of the sample (Fig... Using 30 min at 60 °C the apparent m... weights for these substances in different runs

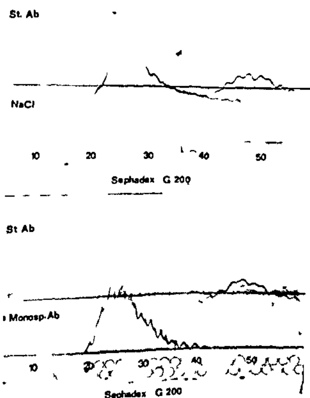
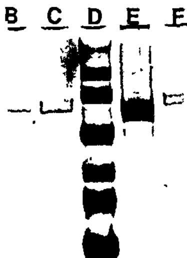


Fig 2 Fused rocket immunoelectrophoresis with i... mediate gel of some of the fractions of Na_2SO_4 Ag e... from a Sephadex G 200 column (2 5 × 120 cm) first 130 ml of the eluant fluid was discarded collected fractions were 4 ml each

To each well was applied 5 µl of the fractions ind... on the figure

A 30 µl/cm² of the polyspecific St Ab in the se... dimension gel and no antibody in the intermediate g...

B 30 µl/cm² of the polyspecific St Ab in the se... dimension gel and 30 µl/cm² of the monospecific Ca in the intermediate gel A large precipitate corres... ding essentially to the 17 fractions 20–36 has drawn into the intermediate gel and correspond... Common Antigen This antigen is the first one e... from the column Blue dextran of a mean mol wt of 10^6 Dalton was eluted in the same fractions as C... Antig...



by salting-out and G-200 filtration The concentration of CA was estimated by measuring the heights of rockets as compared to varying concentrations of crude extract Na_2SO_4-Ag contained 32 3% of the protein of crude extract and 50% of the CA Whereas the relative total protein concentration was rather constant in different preparations of Na_2SO_4-Ag, the relative concentration of CA varied between 50–75% The mean protein concentration of the 17 G-200 fractions which contained CA was 0 055 mg/ml corresponding to 8 8% of the protein applied to the column The concentration of CA was 5 1% of that of crude extract or 10 2% of that of Na_2SO_4 Ag Taking the 6 8 time increase in volume into account the revovery of CA from the column was about 70% or 35% of the crude extract Table I also shows that in the fraction with peak concentration of CA the relative purity of CA was 15 8 times greater than in the crude extract

ɔlyacrylamide-electrophoresis with dodecyl fate A and D protein markers B C E and

ion with sample buffer at room temperature

30 min
15 min
10 min
ical details see text

ns in E were calculated to 59 700 and 54 000 ly

ed to 62–65000 and 59–62000 Dalton ively No other bands were observed on the AGE gel When a trail of SDS PAGE with aterial was used for the first dimension gel of 1 immunoelectrophoresis with anti-CA a itate with two peaks corresponding rather

DISCUSSION

Common antigen from *P aeruginosa* seems to be the bacterial antigen with the widest spectrum of cross reactivity hitherto described CA corresponds to the High Mobility Antigen from *E coli* (6) described by *Kayser* (14) but its biochemical properties differ from those of Enterobacterial Common Antigen (16) or Pseudomonas Common Antigen (13) *Høiby* (7) reported cross reactivity of CA with antigens from *Diplococcus pneumoniae Flavobacterium meningosepticum Bacillus cereus Neisseria meningitidis Haemophilus influenzae Bordetella pertussis Pasteurella multocida Escherichia coli Salmonella typhi Shigella sonnei Citrobacter intermedius Enterobacter cloacae Klebsiella pneumoniae Serratia marcescens Proteus mirabilis Yersinia enterocolitica Vibrio cholera* and several Pseudomonas species The reference system of *Bordetella pertussis* had a rather similar spectrum of CA (12), whereas the corresponding *N meningitidis* antigen (no 19 in ref 6) in addition cross reacted with strains of *Bacillus subtilis Corynebacterium* sp *Clostridium perfringens* and *Neisseria catarrhalis* In neither of the organisms mentioned was cross reaction with *Bacteroides fragilis Staphylococcus aureus* or *Streptococcus pyogenes* observed Later studies showed no cross reaction with *Mycobacterium phlei M intracellulare* an Actinomycetes sp or an Aspergillus sp whereas both an Azotobacter sp and *Campylobacter fetus* cross reacted (unpublished results J B H and Z S) It should be emphasized that in most cases only a single strain from each taxonomic group was

TABLE 1 *Isolation of Common Antigen Concentration of Protein and Common Antigen in Various Frac*

	Vol in ml	Protein (mg/ml)	Concentration in % of crude extract	Common Antigen Arbitrary Units per ml	per mg protein	% frac
Crude French Press extract of *Pseudomonas aeruginosa*	10	14 7	–	100	6 80	10
Na_2SO_4-Ag[1]	10	4 75	32 3	50	10 53	5
Pooled fractions from Sephadex G-200 chromatography[2,3]	68	0 055	0 37	5 1	92 7	[illegible]
Fraction 24 from Sephadex G-200 chromatography	4	0 126	0 86	13 5	107 14	[illegible]

1) Supernatant after precipitation of crude extract with 18 % Na_2SO_4
2) The examinations were performed with a mixture of equal volumes of the 17 fractions that contained CA
3) See Fig 3

each (Fig 2) Ciwig with individual fractions of antigen in the first dimension and with St-Ab in the second dimension gel indicated that 9 of the 17 fractions, including those with peak concentration of CA were immunoelectrophoretically pure and in the other 8 fractions only one additional precipitating antigen was demonstrated When a pool of all 17 fractions was applied to a column of A25 DEAE and eluted with a gradient of NaCl phosphate buffer, CA appeared in the fractions 0 3–0 4 M NaCl and no other antigen demonstrated in these fractions

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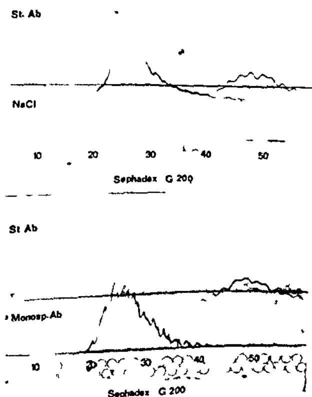


Fig 2 Fused rocket immunoelectrophoresis with intermediate gel of some of the fractions of Na_2SO_4-Ag from a Sephadex G 200 column (2 5 × 120 cm) first 130 ml of the eluant fluid was discarded collected fractions were 4 ml each

To each well was applied 5 µl of the fractions indicated on the figure

A 30 µl/cm² of the polyspecific St Ab in the second dimension gel and no antibody in the intermediate gel

B 30 µl/cm² of the polyspecific St Ab in the second dimension gel and 30 µl/cm² of the monospecific Ca in the intermediate gel A large precipitate corresponding essentially to the 17 fractions 20–36 has been drawn into the intermediate gel and corresponds to Common Antigen This antigen is the first one eluted from the column Blue dextran of a mean mol wt of 2 10⁶ Dalton was eluted in the same fractions as Common Antigen

y N & Axelsen N H Identification and ...ntation of precipitins against *Pseudomonas* ...ginosa in patients with cystic fibrosis by means ...ossed immunoelectrophoresis with intermediate Acta path microbiol scand Sect B *81* 298– 1973
...y N Hertz J B & Andersen V Cross ...ions between *Bordetella pertussis* and twenty ... other bacterial species Acta path microbiol ...d Sect B *84* 395–400 1975
...ma J Y Abe C Tanamoto K Hirao Y ...hara K Tsuzuki H Yanagawa R Honda ...tor Y Fujimoto Y Gonio M Imaeki N ...a H Goda A Takeuchi S & Ishihara T ...ctiveness of immunization with single and ...ti-component vaccines prepared from a Com... antigen (OEP) protease and elastase toxoids of ...domonas aeruginosa and protection against ...orrhagic pneumonia in mink due to *P aerugi*... Jap J Exper Med *48* 111–133 1978
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examined In spite of this the above list of cross-reactions is most impressive

The present study on the purification of CA was attempted as a part of a project on the biochemical characterization (22) and a study of the physiological function of this substance By affinity chromatography with antibody coupled to the matrix for the purification of CA a good yield was obtained by elution with SDS-containing buffer (unpublished observations), however, SDS protein complexes are not suitable for some biochemical examinations, and the electrical charge of the eluted protein is altered It was therefore beneficial that CA proved relatively easy to purify from the crude bacterial extract by a combination of two simple procedures Precipitation of unrelated antigens with 18% Na_2SO_4 followed by gel filtration on a column of Sephadex G-200 Furter purification was obtained by desorption from an anion exchange gel (A25-DEAE) The total yield of purified CA was 35% of the content in the crude bacterial extract CA proved to be the antigen of the highest mol wt of all antigens not salted out by Na_2SO_4 CA was eluted with the void volumen of the G 200 gel indicating a molecular weight of > 4–600 000 SDS-PAGE showed that it could be dissociated into subunits with molecular weight of 62–65 000 and 59–62 000 Dalton It is not known whether these bands represent different degrees of degradation of identical subunits or two subunits of different primary structure

The fractios with the highest concentration of CA from the Sephadex G 200-column (2 5 × 120 cm) seemed to be immunochemically pure For example Ciwig of fraction 24 (see Table 1) against the polyspecific St-Ab did not reveal any other precipitate than that of the Common Antigen The Ciwig was performed with 5 µl antigen in the first dimension gel or 630 ng protein (Table 1) Provided this protein is pure and CA contains only protein then each ml of crude extract contains

$$\frac{100}{13\ 5} \times 0\ 126 = 0\ 93 \text{ mg}$$

Common Antigen per ml or approximately 6% Anyhow this estimate is provisional and awaits further support by additional techniques

The technique for isolation of CA by Na_2SO_4 precipitation combined with G-200-chromatography seems satisfactory for preparative purposes The loss of CA by salting out is of less importance since the prodedure is cheap rapid and simple The recovery of CA from Sephadex gel filtration is high and its purity in most of the fractions will probably prove sufficient for further biochemical analyses

This investigation was supported by grants to D S the *Danish Ministry of Education* and to J B H L *Danish Medical Research Council* We also wish *M Weis Bentzon* Actuary Statens Serumins help with statistical analyses and Mrs *Ann Be* brilliant technical assistance D S is established gator of the Head Scientist s Bureau The Min Health Israel

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ULTRASTRUCTURAL OBSERVATIONS ON MICROGAMETOGENESIS AND THE STRUCTURE OF THE MICROGAMETE OF *ISOSPORA FELIS*

D J P FERGUSON[1,2*] A BIRCH ANDERSEN[2] W M HUTCHISON[3] and J CHR SIIM[1]

FAO/WHO Collaborating Centre for Research and Reference in Toxoplasmosis[1] and Department of Biophysics[2] Statens Seruminstitut Copenhagen Denmark and Department of Biology[3] University of Strathclyde Glasgow Scotland

Ferguson D J P Birch Andersen A Hutchison W M & Siim J Chr Ultrastructural observations on microgametogenesis and the structure of the microgamete of *Isospora felis* Acta path microbiol scand Sect. B 88 151–159 1980

The endogenous forms of *Isospora felis* were observed within the epithelial cells of the small intestine of the cat They were situated within a parasitophorous vacuole which was limited by a multimembranous wall The ultrastructural features of microgametogenesis were studied at 8 days post infection The initial phase of microgamont development consisted of cytoplasmic growth accompanied by a number of nuclear divisions The gamont was enclosed by a pellicle and its surface area was greatly increased by deep invaginations In the later stages of development the numerous nuclei were situated close to the pellicle Each nucleus has peripherally condensed chromatin Formation of the microgametes occurred as protrusions from the microgamont surface Two basal bodies the dense portion of a nucleus and a mitochondrion entered each protrusion The microgametes matured while still attached to the gamont from which they finally budded off into the parasitophorous vacuole leaving a large residual cytoplasmic mass The mature microgamete was found to consist of an elongate nucleus which overlaps with a mitochondrion towards the anterior end of the organism The anterior portion contains a dense perforatorium and two basal bodies with attached flagella In addition a number of microtubules (5–9) were found to run longitudinally from the basal body region

Key words *Isospora felis* ultrastructure microgametogenesis microgamont cat

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Received 8 xi 79 Accepted 25 i 80

Within the coccidia there have been few reports of the ultrastructure of the endogenous forms of members of the genus *Isospora* (13 22) Recent discoveries have shown that *Toxoplasma gondii* *Sarcocystis* spp *Besnoitia* spp and *Frenkelia* spp are coccidian parasites which produce *Isospora* like oocysts (for references see 1 5 6 15)

* Danish Medical Research Council Fellow Present address Department of Pathology University of Edinburgh Medical School Teviot Place Edinburgh Scot

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To the best of our knowledge there have been no reports on the fine structure of microgametogenesis in the genus *Isospora* except for a few observations on the immature microgamont of *I belli* (22) In the present paper we describe the host/parasite relationship the process of microgametogenesis and the structure of the microgamete of *I felis*

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In two recent reviews on the classification of the coccidia it has been proposed that certain species including *I felis* should be removed from the genus *Isospora* and placed in a new genus Two names have been proposed for this genus *Levineia* (1) and *Cystoisospora* (5) Since neither of these names have been generally accepted we will in this report to prevent any confusion refer to the organism studied as *Isospora felis*

MATERIALS AND METHODS

Specific Pathogen Free cats were infected using sporulated oocysts of *I felis* administered via a stomach tube To study microgametogenesis the cats were autopsied at 8 to 9 days post infection The small intestine was removed and divided into 12 portions Samples from the portions with the highest parasite density were processed for electron microscopy The procedure used was similar to that described previously for *Eimeria brunetti* (2) In summary the samples were fixed in glutaraldehyde and osmium tetroxide and embedded in either Vestopal W or Spurr s epon Thin sections were examined in a Philips EM 200 or EM 300 electron microscope after staining with uranyl acetate and lead citrate The results are based on the examination of more than 400 micrographs

RESULTS

Host/Parasite Relationship

The infecting organisms were situated in a parasitophorous vacuole within the epithelial cells of the small intestine This vacuole lay between the nucleus and the microvilli of the brush border of the host cell The limiting wall of the vacuole was 30 nm thick and consisted of four electron dense and three electron lucent bands (Figs 2 4) It appear that membranes of the host cell reticulum are involved in the formation of (Fig 2) and its substructure is consistent close aposition of three unit membranes

As the parasites grew the host cell distended In addition the host cell n nucleolus were greatly enlarged (Fig 13)

During parasite development small amorphous material (approximately 60 diameter) were observed within the pa vacuole adjacent to its limiting wall The vacuole had formed evaginations into the cytoplasm and a number of the amorphous were present in these evaginations (Figs Parasitophorous vacuoles containing micr possessed fewer and shorter evaginations which contained macrogamonts (cf Figs 1

Microgametogenesis

Within the epithelial cell the infecting mero underwent dedifferentiation with the loss of anterior organelles and thus formed a trop Microgametogenesis can be divided into two The initial phase involves cytoplasmic accompanied by numerous nuclear divisions reby large multinucleate microgamonts were ced The nuclei were evenly distributed in cytoplasm and each nucleus had a distinct n and well dispersed chromatin (Fig 1) The n divisions involved an eccentrically located with associated centrioles The cytoplasm of microgamont contained a number of Golgi some swollen mitochondria dense bodies and strands of rough endoplasmic reticulum

Figs 1–13 are electron micrographs of sections which represent stages in the process of microgametogenesis show the ultrastructure of the microgamete of *Isospora felis*

A double bar (=) on a micrograph represents 1 µm and a single bar (–) 100 nm

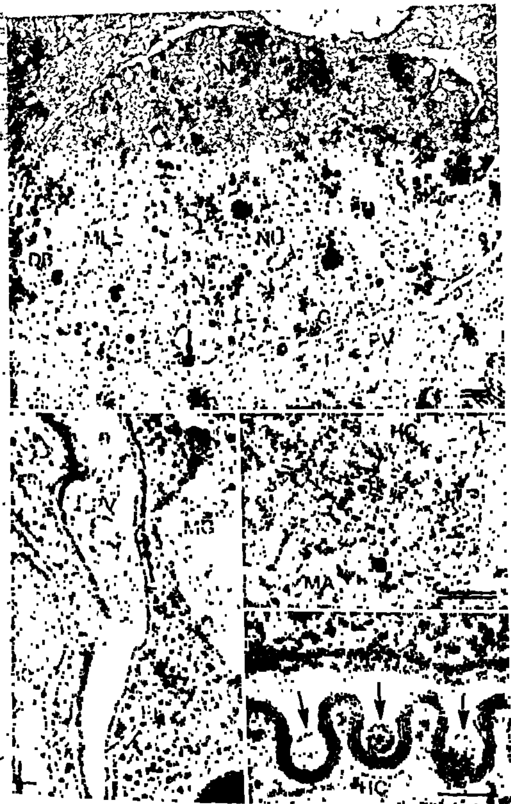
The following abbreviations are used throughout B = basal body CE = centriole DB = dense body ER rough endoplasmic reticulum FL = flagellum G = Golgi body HC = host cell HN = host nucleus MA macrogamont MG = microgamont MI = mitochondrion N = nucleus NU = nucleolus PG = polysa granule PL pellicle PV = parasitophorous vacuole RM = residual cytoplasmic mass RN = residual n

Fig 1 An early microgamont which possesses a number of evenly dispersed nuclei The cytoplasm also con number of dense bodies mitochondria and Golgi bodies Note the number of short invaginations of the (arrows) × 7 500

Fig 2 In this section the periphery of a microgamont and part of the host cell are illustrated The microgam limited by a pellicle Note that the third unit membrane involved in the formation of the multilayered wall of parasitophorous vacuole is not closely applied to the other two membranes in certain regions (arrows) × 90

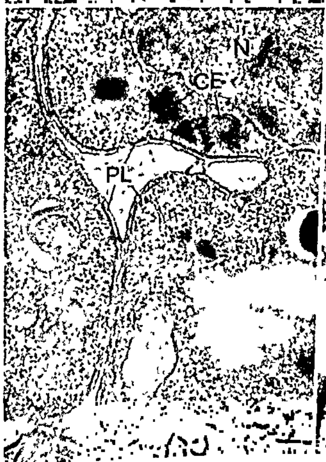
Fig 3 A section through part of a host cell containing a macrogamont showing the numerous deep invaginations the wall of the parasitophorous vacuole (arrows) which contain patches of amorphous material × 15 000

Fig 4 A high magnification of the wall of a parasitophorous vacuole showing that it consists of four electron and three electron lucent bands Note the evaginations of the wall each of which contains a patch of amorp material (arrows) × 160 000


DB
ML
NU
N
G
PV
MG
HC
MA
HC


5
N
MJ
N
N
DB


6
N
N
N


7
N
CE
PL


8
MI
B

B
N
10
N
N
FL
M
N
FL
Mi
N

Fig 5 A section through part of a microgamont in which a deep invagination of the pellicle is illustrated (a
Note that the nuclei are present close to the pellicle or its invaginations × 7,500

Fig 6 This section is through part of a microgamont at a later stage of development than in Fig 5 Note th
peripherally located nuclei have the chromatin concentrated into a single dense mass × 15 000

Fig 7 A higher magnification of part of an organism at a similar stage to that in Fig 5 showing that the invag
are limited by a pellicular structure Two centrioles situated between a nucleus and the pellicle are also sh
× 45,000

Fig 8 A higher magnification of part of a microgamont at a later stage than that in Fig 6 A basal body from wh
flagellum is protruding into the parasitophorous vacuole is seen At this stage the chromatin is present as a dens
at the side of the nucleus adjacent to the basal body × 45 000

Fig 9 A longitudinal section through two developing microgametes which are still attached by their anterio
the microgamont Note the collar of dense material at each point of attachment (arrows) × 45 000

Fig 10 A section through the anterior part of a microgamete The presence of a dense plaque which consis
number of microtubules embedded in a dense matrix is shown (arrows) The two basal bodies with attached fla
are also illustrated × 90 000

Fig 11 A cross-section through two microgametes at the level of the mitochondrial/nuclear overlap N
microtubules situated round the periphery of each mitochondrion (arrows) × 90 000

Fig 12 A cross-section through the nuclear region of a number of microgametes Note the presence of
microtubules in each organism at this level × 90 000

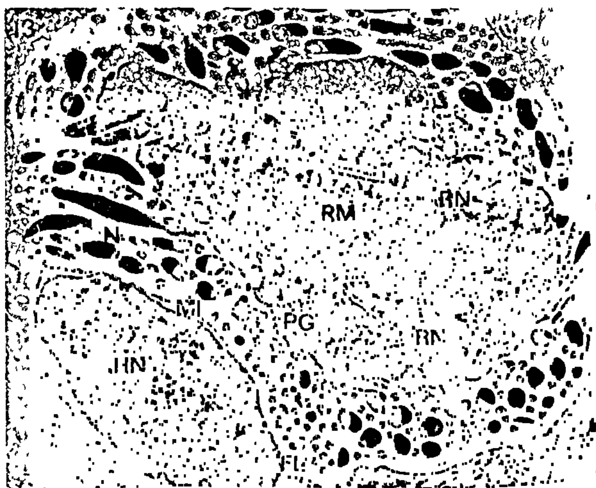


Fig 13 A section through a mature microgamont showing the numerous microgametes within the parasitop
vacuole and the large residual cytoplasmic mass which contains a number of residual nuclei and polysacch
granules × 7 500

large number of free ribosomes (Fig 1) ...out this development the organism remain...ed by a pellicle consisting of an outer unit ...ne and an inner layer of two closely applied ...mbranes (Fig 2) At this multinucleate stage ...icle possessed a few micropores*) and a ... of invaginations had started to develop (Fig

...he later stages the surface area of the ...m was greatly increased by deep invagina... f the pellicle (Fig 5) The final nuclear ...s produced smaller nuclei which lacked a ...us and had the chromatin aggregated into a ...ge dense patches at the periphery (Figs 5 6) ...tion it was noted that the nuclei were ...l close to the pellicle (Figs 5 6) Normally ...triołes were present between the nuclei and ...mbranes of the pellicle (Fig 7)

...second phase of microgametogenesis consist... ...he actual formation of the microgametes The ...entrioles at each nucleus appeared to be ...rmed to basal bodies from which the flagella ...ped (Fig 8) Each flagellum then grew out ...e parasitophorous vacuole (Fig 8) At this ...the inner layer of the pellicle had started to ...rate As this occurred the chromatin was

...ogamete formation now continued by a ...sion of the limiting membrane of the ...gamon...

...n remained in the cytoplasmic mass as a

...still attached by its anterior end to the ...ogamont (Fig 9) At this stage a number of ...otubules and a dense perforatorium were ...rved within the developing microgamete ...t maturity the microgametes budded off into ...parasitophorous vacuole and a large residual ...plasmic mass which contained a number of ...saccharide granules was left behind (Fig 13)

Each microgamont was very large and could give rise to several hundreds of microgametes

The mature microgamete was crescentic in shape and consisted of a dense elongated nucleus which showed overlapping with a mitochondrion towards the anterior end of the organism (Figs 9 11) The nucleus was enclosed by two closely applied membranes and the mitochondrion possessed several rows of bulbous cristiae The anterior part of the organism consisted of two basal bodies which were embedded in a dense matrix and to each of which a flagellum was attached (Fig 10) The two flagella left the microgamete close to the anterior end A dense plaque probably the perforatorium was found close to the anterior tip and in certain sections it was possible to show that it consisted of a number of microtubules embedded in a dense matrix (Fig 10)

A number of microtubules appeared to originate in the basal body region and were observed to run longitudinally through the microgamete They were distributed around the mitochondrion and their number varied between 5 and 9 in the region of the mitochondrion/nuclear overlap (Fig 11) Normally only two microtubules appeared to extend to the posterior tip of the microgamete (Fig 12)

DISCUSSION

The host/parasite relationship observed in our study of *I felis* is similar to that reported by *Pelster* (13) Also the multi membranous structure of the wall of the parasitophorous vacuole is similar to that described for the endogenous forms of *T gondii* (14) *I rivolta* (13) and *S fusiformis* (18) This is in contrast to the vacuole enclosing the endogenous forms of *Eimeria* spp which is limited by a single unit membrane (2 12) The involvement of the endoplasmic reticulum of the host cell in the formation of the parasitophorous vacuole in *I felis* is similar to that reported for *S fusiformis* (18) and *S suihominis* (11) The functional significance of the patches of dense material present within the evaginations of the parasitophorous vacuole is unknown

In the microgametogenesis of *I felis* the initial growth phase with nuclear division and chromatin changes is similar to that described for *Eimeria* spp (3 7 10 17 19 20 21 23) *T gondii* (4 14) *Sarcocystis* spp (24 25) and *I belli* (22) However in the case of *S suihominis* studied in vitro nuclear division did not occur prior to the initiation of microgamete formation (11) A retension of the intact pellicle was observed for the developing microgamont of *I felis* This is different from what

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Fig 5 A section through part of a microgamont in which a deep invagination of the pellicle is illustrated. Note that the nuclei are present close to the pellicle or its invaginations × 7,500

Fig 6 This section is through part of a microgamont at a later stage of development than in Fig 5 Note the peripherally located nuclei have the chromatin concentrated into a single dense mass × 15 000

Fig 7 A higher magnification of part of an organism at a similar stage to that in Fig 5 showing that the invag are limited by a pellicular structure Two centrioles situated between a nucleus and the pellicle are also s × 45,000

Fig 8 A higher magnification of part of a microgamont at a later stage than that in Fig 6 A basal body flagellum is protruding into the parasitophorous vacuole is seen At this stage the chromatin is present as a dens at the side of the nucleus adjacent to the basal body × 45 000

Fig 9 A longitudinal section through two developing microgametes which are still attached by their anterior the microgamont Note the collar of dense material at each point of attachment (arrows) × 45 000

Fig 10 A section through the anterior part of a microgamete The presence of a dense plaque which consis number of microtubules embedded in a dense matrix is shown (arrows) The two basal bodies with attached are also illustrated × 90 000

Fig 11 A cross section through two microgametes at the level of the mitochondrial/nuclear overlap microtubules situated round the periphery of each mitochondrion (arrows) × 90,000

Fig 12 A cross-section through the nuclear region of a number of microgametes Note the presence of microtubules in each organism at this level × 90 000

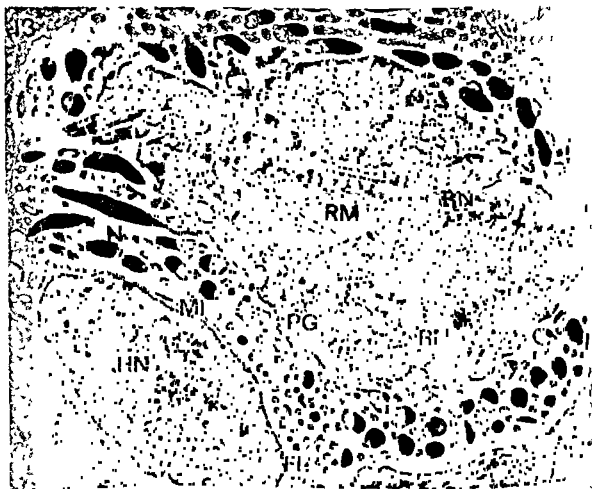


Fig 13 A section through a mature microgamont showing the numerous microgametes within the parasit vacuole and the large residual cytoplasmic mass which contains a number of residual nuclei and polysacch granules ×

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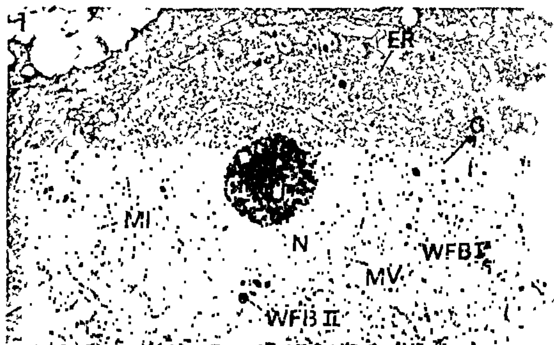
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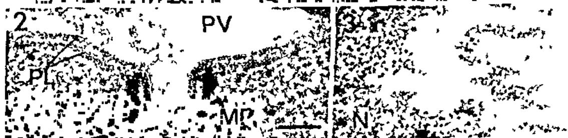
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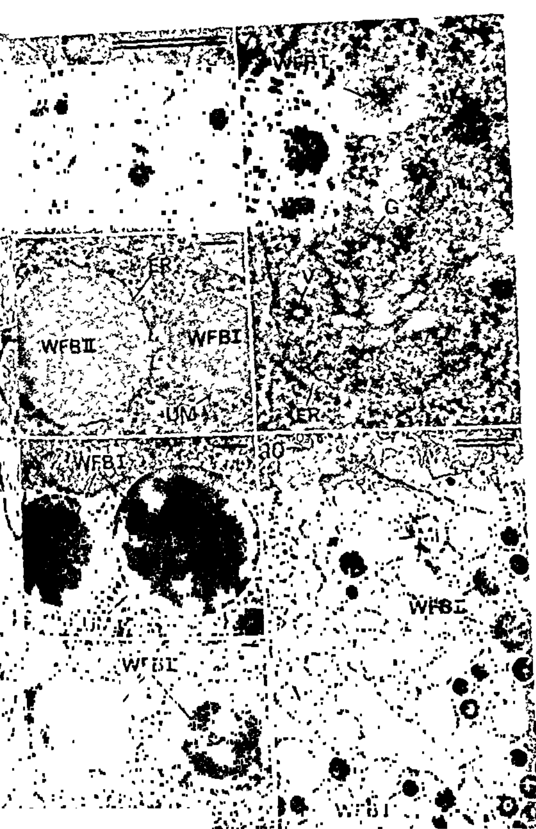
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1
ER
G
MI
N
WFB I
MV
WFB II


2
PV
PL
MP
3
N


WFBI
G
ER
WFBII
WFBI
UM
V
ER
WFBI
WFBI
WFBI

11

WFB II

L

L

L

2

L

N

NU

WFB I

WFB II

PV

MI

PG

-12 are electron micrographs which illustrate various stages in the process of macrogametogenesis and the ucture of the macrogamete of *Isospora felis*
le bar (=) on a micrograph represents 1 μm and a single bar (–) 100 nm
lowing abbreviations are used throughout C = canaliculi ER = rough endoplasmic reticulum G = Golgi HMI – host cell mitochondrion HN = host cell nucleus L = lipid globule MI = mitochondrion MP = ore MV = multimembranous vacuole N = nucleus NU = nucleolus PG = polysaccharide granules PL cle PV – parasitophorous vacuole UM = unit membrane V = coated vesicle WFB I = wall forming f type I WFB II = wall forming body of type II

A longitudinal section through an early macrogamont showing the large nucleus and nucleolus The cytoplasm is a number of peripherally located mitochondria Golgi bodies and amounts of rough endoplasmic reticulum stages in the formation of WFB I and WFB II are also shown × 7 500

A high magnification of a section through the periphery of a macrogamont in which the structure of the pellicle s adaptation to form a micropore can be seen × 90 000

A higher magnification of part of the organism shown in Fig 4 Note the multimembranous vacuole which to bud off from the nucleus (arrow) × 45 000

A section through a macrogamont in which the cytoplasm contains several immature WFB I and II a number ochondria Golgi bodies polysaccharide granules lipid globules and multimembranous vacuoles The nucleus a large nucleolus is also shown × 7 500

A section through part of a macrogamont showing two Golgi bodies with associated membranes of the rough lasmic reticulum Coated vesicles are present within the Golgi body on the left while a WFB I can be seen at the of the Golgi at the right An immature WFB II is illustrated within the cisterna of the rough endoplasmic ilum × 30 000

5 Immature WFB I and II are illustrated in this micrograph The WFB II has an amorphous appearance and is ted within a cisterna of the rough endoplasmic reticulum while the WFB I is limited by a unit *membrane* 000

7 This section illustrates a Golgi body which apparently is involved in formation of WFB I A *membrane of* h endoplasmic reticulum is present at the base of the Golgi body and a coated vesicle is also observed in this n At *the apex of the Golgi* body a number of large vacuoles are seen which contain material similar to that n WFB I × 90 000

8 A section through two mature WFB I showing that each is limited by a unit membrane In this case the WFB I lay a mottled appearance towards the periphery × 90 000

9 *In this section* through two mature WFB II it can be seen that they possess an amorphous core with a more ular cortex Note also that each is situated within a cisterna of the rough endoplasmic reticulum × 45 000

10 A section *through* part of a *late* macrogamont in which a deep invagination of the pellicle is present (arrow) 5 000

11 In this section *through part of a late* macrogamont a group of lipid globules can be seen surrounded by a nber of WFB I WFB II and polysaccharide granules × 15 000

12 A section through part of a *mature macrogamete* At this stage the cytoplasm is filled with numerous ysaccharide granules lipid globules WFB I and WFB II *A few mitochondria are also present* Note the smooth line of the organism × 7 500

In the development of the trophozoite into the icrogamont cytoplasmic growth was *not* accom nied by nuclear division and the early m ...

... the organism was found to be limited by a pellicle which possessed a few micropores (Fig 2) The cytoplasm of the early macrogamont contained a number of peripherally located mitochondria numerous parallel strands of rough endoplasmic reticulum (rER) *many free* ribosomes as well as polyribosomes and a *number* of Golgi bodies (Fig 1)

At an early stage while the organism was still ellipsoidal in shape *the formation* of the wall forming bodies of types I and II (WFB I and WFB

11

WFB II

L

L

L

2

L

N

Nu

WFB I

G

WFB II

L

L

PV

MI

PG

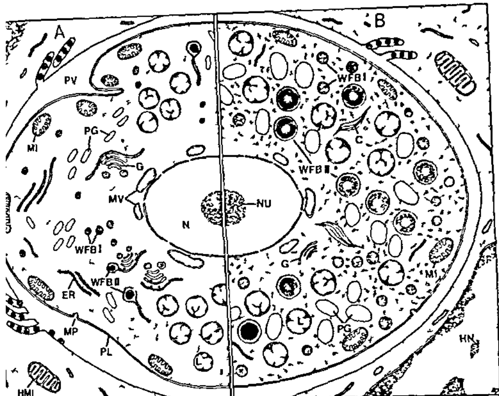


Fig 1 A diagramatical representation of the ultrastructure of part of an early macrogamont is given in part A and that of a mature macrogamete in part B

ished micrographs it would appear that the electron lucent wall forming bodies are more likely to represent reserve material (lipid or polysaccharide)

For the coccidia the method of formation of the WFB is incompletely understood However our results on I felis are able to provide some morphological information It appears that the early macrogamont of I felis is well equipped for protein synthesis with large amounts of rER and numerous ribosomes and polyribosomes Our observations on the formation of the WFB I would be consistent with the passage of protein from the rER via the coated vesicles (transfer vesicles) to the Golgi body where it is linked to other substances to produce the material of the WFB I Such a pathway of synthesis is similar to that previously proposed in ... (20) and is compatible with earlier observations in T gondii (6) As pointed out by Wheat et al (20) such a process could result in the WFB I containing protein carbohydrate complex which is in concordance with the chemical nature of the outer layer of the oocyst wall which is formed by the WFB I (13) The WFB II are formed within the cisternae of the rER This is similar to that reported for *Eimeria* spp (1 2 4 8 15 16 18) and *T gondii* (6 12) and probably represents the precipitation of protein molecules formed by the membrane bound ribosomes The presence of a core and cortex appearance in the mature WFB II is similar to that observed in *I felis* and *I rivolta* (11) Although a Golgi body is observed close to the developing WFB II it does not appear to be intimately involved in WFB II formation This observation differs from that previously reported for *E brunetti* (4) and *E acervulina* (7)

We are indebted for Mr *J F Dunache* and Miss *M Reilly* for the maintenance of the SPF cats We gratefully acknowledge Mrs *H Ran* and Mrs *J Berg* for technical assistance and Miss *A G Ostergaard* and Mr *F Laursen* for photographic assistance The work was supported by grants from the *WHO* Geneva the *Wellcome Trust* the *Danish Medical Research Council* and the *W R Ross Foundation*

II) was initiated simultaneously in the cytoplasm (Fig 1) The rER and Golgi bodies seemed to be involved in the formation of the WFB I (Figs 1 4) Generally a strand of rER was observed close to the base of a Golgi body (Figs 5 7) The portion of ER adjacent to the Golgi body had lost the ribosomes and a few coated vesicles were present in this region of the Golgi (Figs 5 7) Vacuoles were now observed at the anterior part of the Golgi These contained material similar to that within the WFB I and small WFB I were also present close to the Golgi bodies (Figs 5 7) The WFB I seemed to mature into structures approximately 400 nm in diameter (D) with dense amorphous contents and they were limited by a unit membrane (Fig 8) In a few cases they displayed a mottled appearance towards the periphery (Fig 8)

The WFB II were first observed as amorphous spheres 150 nm D within the cisternae of the rER In many cases a Golgi body was found to be situated close to the rER (Figs 4 6) The WFB II continued to mature within the cisternae of the rER where the largest could reach a size of 900 nm D (Fig 9) The mature WFB II situated within distended cisternae of the rER showed a fine amorphous core with a more granular cortex (Fig 9)

At an early stage of macrogametogenesis changes were observed at the periphery of the nucleus where a number of portions of nuclear membrane and nucleoplasm appeared to be budded off to form multimembranous vacuoles (Fig 3) These vacuoles contained electron lucent material and remained situated close to the nucleus (Figs 1 4)

Within the developing macrogamont a store of reserve material in the form of lipid globules and polysaccharide granules was produced (Fig 4) The lipid globules were the first to mature and could reach approximately 1 2 µm D (Fig 11) They were normally observed to be situated in small clusters in the cytoplasm of the macrogamont (Fig 11) The polysaccharide granules first appeared as small rod shaped structures (Figs 4 11) but as the macrogamont matured they became more spherical in appearance and increased in size reaching 1 2 µm D (Fig 12)

During the process of macrogametogenesis the number and size of the WFB I and II the polysaccharide granules and lipid globules increased (Fig 11) During this development the macrogamont presented an irregular outline with a number of invaginations of the pellicle (Fig 10)

The mature macrogamete was spherical in appearance with a smooth outline and limited by a pellicle The organism possessed a large nucleus with a nucleolus and the cytoplasm was packed with numerous WFB I WFB II polysaccharide granules and lipid globules (Fig 12) A number of [illegible] membranous vacuoles were observed close [illegible] nucleus and a few canaliculi Golgi bodies [illegible] chondria and a little rER were present [illegible] cytoplasm (Fig 12)

Text Fig 1 gives a diagramatical repres[illegible] of the ultrastructure of part of an early ma[illegible] mont (A) and part of a mature macrogam[illegible]

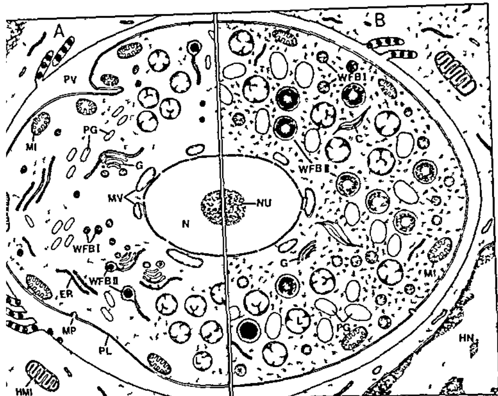
DISCUSSION

The host/parasite relationship of the macro[illegible] of *I felis* as observed in the present study is [illegible] to that reported previously (11) It should be [illegible] that the parasitophorous vacuoles lack intra[illegible] tubules This is also the case in *Toxoplasma* [illegible] (6 11 12) *I rivolta* (11) *Sarcocystis* spp (19) *Eimeria magna* (16) and *E ferrisi* (11) [illegible] intravacuolar tubules are thought to be a chara[illegible]ristic feature of the macrogametes of certain [illegible] *Eimeria* spp (see ref in 10 and 15)

The formation of multimembranous va[illegible] from the nuclear surface in *I felis* has a[illegible] reported for *T gondii* (6) and *E m[illegible]* Vacuoles with a similar appearance have also [illegible] observed close to the nucleus in a number [illegible] *Eimeria* spp (4 7 8 16 18) It is likely that th[illegible] vacuoles have a common function in the va[illegible] species but the function is unknown The va[illegible] are also similar in appearance to the 'dark bo[illegible] which have been reported as a new feature [illegible] macrogamete of *Sarcocystis fusiformis* (14)

Our observation that the reserve material in [illegible] macrogamete of *I felis* consists of lipid globules [illegible] addition to polysaccharide granules differs from [illegible] of *P elster* (11) where only polysaccharide gra[illegible] were noted The development of large amou[illegible] reserve material is also characteristic of the pr[illegible] of macrogametogenesis in *Eimeria* spp (1 2 [illegible] 15 16 18) in *Sarcocystis* spp (14 21) and [illegible] *rivolta* (11) These large amounts of food reser[illegible] are probably necessary to meet the energy req[illegible]ments of the process of sporulation

Within the coccidia the most important chara[illegible]ristic of the macrogamont is the ability to synth[illegible] the wall forming bodies which give rise to [illegible] resistant oocyst wall The macrogametes of *I f[illegible]* are similar to those of *Eimeria* spp (1 2 3 4 8 [illegible] 16 18) *T gondii* (6 12) *I rivolta* (11) [illegible] *suihominis* (9) and *S fusiformis* (14) in that [illegible] types of WFB are present However in *Sarcoc[illegible]* sp (19) and *S singaporensis* (21 22) only one [illegible] of body has been observed For *I belli* the situa[illegible] is unclear Here two types of WFB have be[illegible] reported (17) b[illegible] a close e[illegible]ation of



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INHIBITION OF HEPATITIS B DANE PARTICLE DNA)LYMERASE ACTIVITY BY PYROPHOSPHATE ANALOGS

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rdenfelt E Öberg B Helgstrand E & Miller E Inhibition of hepatitis B Dane particle DNA lymerase activity by pyrophosphate analogs Acta path microbiol scand Sect B 88 169–175 1980

DNA polymerase is associated with the core of the so-called Dane particles The probability that this the hepatitis B viral DNA polymerase offers the possibility of preventing hepatitis B multiplication selective inhibition of this enzyme We have previously reported that trisodium phosphonoformate FA) inhibits Dane particle DNA polymerase Fifteen compounds with structural similarity to PFA d pyrophosphate have now been tested for inhibition of hepatitis B virus DNA polymerase in an tempt to define the structural requirement for the inhibition Active structures have two acid groups close proximity of which at least one is a phosphono group Phosphonoformate and hypophosphate ere the two most active inhibitors The K_i value for PFA was 7 2 μM when dTTP was used as ariable substrate and the mechanism of inhibition was non-competitive Phosphonoformate caused a p d shut-off of the polymerase reaction indicating that it might inhibit elongation The efficient hibition of hepatitis B virus DNA polymerase by PFA and its low toxicity suggest that it could be sed to inhibit hepatitis B virus multiplication *in vivo*

ey words Hepatitis B DNA polymerase trisodium phosphonoformate (PFA)

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ridence available indicated that the so-called e particles (6) are the hepatitis B virus A DNA merase is associated with the core of these cles (13 16) The probability that this is the l DNA polymerase (18) offers the possibility of enting hepatitis B virus multiplication by ctive inhibition of the Dane particle DNA merase

ntercalating agents like ethidium bromide have n reported to inhibit hepatitis B virus DNA ymerase activity probably by binding to the A but no selectivity for viral DNA has been wn (11)

n a preliminary communication we have repor that phosphonoformate (PFA) inhibits hepatitis irus DNA polymerase (21) This finding is here extended to a determination of the mechanism of inhibition by PFA and an investigation of the structure activity relation for several PFA analogs These structures can also be regarded as pyrophosphate analogs

MATERIALS AND METHODS

Dane Particles

Serum was taken from patients who are chronic hepatitis B antigen (HBsAg) carriers undergoing dialysis Specimens for DNA polymerase assay were prepared as described earlier (22) In our routine assays 2 ml serum was concentrated to 0 1 ml In most of the assays in this study the serum source has been one patient (Ass) where 40 ml serum was collected and then concentrated to 0 75 ml and stored in 100 μl portions at −20 °C

DNA Polymerase Assay

In the first part of the investigation, assay conditions for the DNA polymerase were as described by *Kaplan et al* (16) with minor modifications (22) To 25 µl test sample 2 5 µl 10% Nonidet P-40, 2 5 µl 3% mercaptoethanol were added and the mixture kept at room temperature for 30 minutes This mixture was then added to 100 µl of a solution of 160 mM Tris pH 7 5, 40 mM $MgCl_2$, 120 mM NH_4Cl, 0 5 mM dATP, 0 5 mM dCTP, 0 5 mM dGTP and 20 pmole (methyl-^{3}H) dTTP (40 Ci/mmole, New England Nuclear Corporation Frankfurt/M) (Method 1) The mixture was incubated at 37 °C, and at indicated times 20 µl samples were spotted on CF/C Whatman glassfiber papers The filters were left in 5% TCA/0 1 M tetrasodium pyrophosphate solution overnight, washed dried and the radioactivity counted

During most of the investigation this method was modified, as suggested by *Hirschman et al* (12) The 120 mM NH_4Cl was replaced by 400 mM KCl Mercaptoethanol was excluded and instead 20 mM dithiothreitol was included in the 100 µl solution added to the sample Finally, as labelled nucleotide 2 5 µl (^{32}P) dTTP (300 Ci/mmol, 1 0 mCi/ml Radiochemical Centre Amersham England) was used instead of (^{3}H)-dTTP (Method 2) These changes gave 10–20 times higher incorporation in cpm compared to the earlier method in tests run in parallel

The inhibitors were added to the reaction mixture at 4 °C before incubation at 37 °C was started In all assays duplicate aliquots were taken and the results given in cpm are the mean of observed cpm

Pyrophosphate Analogs

Tetrasodium pyrophosphate (I) oxalic acid (III) and glycolic acid (VII) were from E Merck Darmstadt West Germany Disodium sulphonoacetate (XV) was from Eastman Kodak Co Rochester N Y Phosphoglycolic acid (XVII) was from Sigma Chem Co St Louis Miss Disodium ethane-1 hydroxy-1 1-diphosphonate (XI) was a gift from The Procter & Gamble Company Cincinnati Ohio Disodium hypophosphate (II) was prepared according to *Genge et al* (9) Trisodium

(XVI) according to *Nylen* (24) and *Arbuson & Dunin* (2)

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Determination of Inhibition Constants

Determination of inhibition constants and ... of inhibition was according to *Cleland* (4 5) A ... programme for plotting regression lines was used

RESULTS

Time Course of the DNA Polymerase Reaction

Figure 1 shows the time course of a ... reaction where (^{32}P)-dTMP incorporation ... studied during a 5-hour period The incorpora... was almost linear for 4 hours, both at a concentra... tion of 0 043 µMdTTP as shown in Fig 1 and ... concentration of 0 986 µMdTTP which was ... highest concentration used in the mechanism ...

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Mechanism of Inhibition by PFA

The rate of inhibition was investigated ... experiments where PFA was added to the reac... mixture 30 minutes after the start of the reacti...

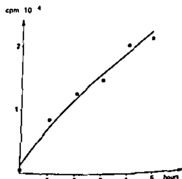


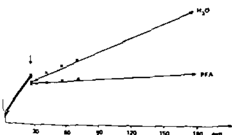
Fig 1 Time-course of the DNA polymerase reaction ... 60 µl of Dane particle preparation 10 µl Nonidet ... and 400 µl of a reaction mixture (according to meth... was added At the designated times after incubation 20 µl aliquots of the reaction mixture were removed ... radioactivity couting

1 *Inhibition of DNA Polymerase Activity by PFA* *Dane Particle Preparations from 5 Patients*

Incorporation in the absence of PFA[b]	100 μM PFA Percentage inhibition	10 μM PFA Percentage inhibition
7087	89	60
14894	90	61
1150	77	42
584	79	47
4738	80	39

5 μl serum preparation was added 15 μl PFA or and 2 5 μl 10 % Nonidet P 40 immediately before g 100 μl solution (according to method 2)
after 3 hours incubation at 37 °C

own in Fig 2 PFA caused an immediate shut the polymerase activity Fig 3A shows the eaver Burk plot for inhibition of hepatitis B DNA polymerase activity by PFA at varying ntrations of dTTP The result indicates that echanism of inhibition is non-competitive As n in Fig 3B a replot of the intercepts and gave the inhibition constants K_i = 7 2 μM 35 0 μM and K_i = 22 6 μM The K_m value dTTP was 0 15 μM as obtained from the eaver Burk plot



2 Rate of inhibition of the DNA polymerase tion by PFA To 60 μl of Dane particle preparation μl Nonidet P 40 and 400 μl reaction mixture (accord to meth ... uba ... re r ... ow ... re a ... liquots were taken immediately before and er the addition of PFA or H_2O The reaction rate is ear both before and after addition Due to the dilution 30 min a lower rate of reaction is observed after that ne

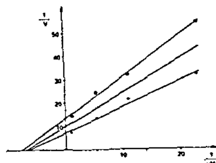


Fig 3A Double reciprocal plots of the hepatitis B virus DNA polymerase-catalyzed reaction with dTTP as variable substrate and PFA as inhibitor The reaction was according to method 2 and the incorporation of (³ P)-dTMP during 4 hours was determined Phosphonoformate concentrations were 0 μM (+) 10 μM (O) and 20 μM (●)

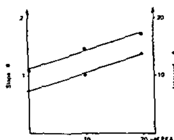


Fig 3B Replots of slopes (●) and intercepts (+) as a function of PFA concentration Date were taken from Fig 3A

Inhibition of Hepatitis B Virus DNA Polymerase by Analogs of PFA

The inhibition of hepatitis B virus DNA polymerase activity by PFA and several analogs of PFA and pyrophosphate is shown in Table 2 A 50% inhibition was observed at 20 μM PFA Hypophosphate (II) inhibited the polymerase activity by more than 90% at 500 μM concentration A slight inhibition was also seen for pyrophosphate (I) and carbonyldiphosphonate (IX)

The rest of the compounds at up to 500 μM concentration did not show any significant inhibition for the DNA polymerase activity

DISCUSSION

The hepatitis B virus DNA in the Dane particles seems to be circular (28) with about two thirds as double and about one third as single-stranded DNA

DNA Polymerase Assay

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ding to *Schwarzenbach & Zurc* (30) tetra sodium ... inehy ... (26) ...) was

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The rate of inhibition was investigated ... experiments where PFA was added to the rea... mixture 30 minutes after the start of the reac...

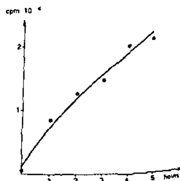


Fig 1 Time course of the DNA polymerase reactio... 60 µl of Dane particle preparation 10 µl Nonidet ... and 400 µl of a reaction mixture (according to met... was added At the designated times after incubatio... 20 µl aliquots of the reaction mixture were remov... radioactivity couting

Compound	Percentage inhibition at 500 µM
HO-P(=O)(OH)-CH₂-CH₂-C(=O)OH	14
HO-P(=O)(OH)-O-CH₂-C(=O)OH	−22

5 µl sem m n a
ion to
before
ances . . . XIII and XVI were tested
d ng to method I and the others according to
xd 2
lots were taken immediately and after 3 hours
ation at 37 °C

The core associated DNA polymerase (13 16) been *reported* to initiate DNA synthesis at om on this *partly* single stranded DNA (19) study of this enzyme and its reaction has so far hampered by the low enzyme activity as mined by *incorporation* of (^{3}H)-labelled nucleo monophosphates and the fact that the Dane cles can only be *obtained* from patient or npanzee sera

few attempts have earlier been made to find itors of the hepatitis B virus DNA polymerase ity Intercalating agents such as ethidium ide and quinacrin can inhibit the polymerase ity (11) However no specificity in binding to titis B virus DNA as compared to cellular A has been shown for these compounds zole monophosphate vidarabine and phospho etate have earlier been reported to be without t on the hepatitis B virus DNA polymerase tion (14 20) In a preliminary comm
ep
tti
y
the . . . polymerase assay the ease in incorporated radioactivity permitted a re activity study of substances similar to

PFA and a investigation of the mechanism of inhibition by PFA

The hepatitis B virus DNA polymerase *reaction* was rapidly blocked by PFA (Fig 2) which might indicate that PFA is an elongation *inhibitor* The mechanism of inhibition by PFA was non *competitive* (Fig 3A) with regard to dTTP This is in accordance with the mechanism of inhibition by PFA of herpesvirus DNA polymerase (27) influ enza virus RNA polymerase (33) and reverse transcriptase (34) PFA had an inh . . .
K
he . . .
and reverse transcriptase from avian myeloblastosis virus with a K_i of 16 µM when the deoxyribo-nucleoside triphosphates were varied

The inhibitory effect of PFA on *Dane particle* associated DNA polymerase seems to be very specific There is a 50% inhibition *of this* polymerase activity at 20 µM PFA Only at 100 times that concentration 2000 µM a 50% inhibition of cellular DNA *synthesis* has been seen (32) Cellular DNA polymerase γ which also has a low Km value for dTTP (31) is not inhibited by 500 µM PFA (B *Eriksson* personal communication 27) No inhibition af bacterial or mycoplasma . . .

. . . associated DNA polymerase from several other tested DNA polymerases (10 27) which are inhibited to the same *extent by PFA and PAA* Reverse transcrip tase is *the only DNA polymerase* yet investigated that shows the same pattern of inhibition (34) as Dane particle DNA polymerase However in contrast to *Dane particle* DNA polymerase *this* enzyme . . .

particle DNA *polymerase*

Two compounds *hypophosphate* (II) and PFA (IV) were effective inhibitors of *hepatitis* B virus DNA *polymerase* Both could be regarded as analogs of . . .

. . . further blocking *any* of the two acidic groups of PFA led to inactive structures (V VI) *This* is in accordance with their lack of effect on herpesvirus DNA *polymerase* (*Eriksson et al manuscript* in preparation) *Phosphonoacetate* (XII PAA) which has the same effect on herpesvirus DNA polymerase as PFA (10) did not inhibit hepatitis B virus DNA *polymerase* despite the structural similarity Of other *tested structures* at

TABLE 2 *Inhibition of Hepatitis B Virus DNA Polymerase Activity by Pyrophosphate Analogs The Com, Shown in Their Acid Forms*

Compound	Percentage inhibition at 500 μM
I HO-P(=O)(OH)-O-P(=O)(OH)-OH	69
II HO-P(=O)(OH)- P(=O)(OH)-OH	97
III HO(O=)C-C(=O)OH	28
IV PFA HO-P(=O)(OH)-C(=O)OH	95
V HO-P(=O)(OCH₃)—C(=O)OH	3
VI HO-P(=O)(OH)-C(=O)OC₂H₅	18
VII HO-CH₂-C(=O)OH	−10
VIII HO-P(=O)(OH)-CH₂-P(=O)(OH)-OH	−28

Compound	[cut off]
IX HO-P(=O)(OH)-C(=O)-P(=O)(OH)-OH	
X HO-P(=O)(OH)-CH(OH)-P(=O)(OH)-OH	
XI HO-P(=O)(OH)—C(OH)(CH₃)—P(=O)(OH)-OH	
XII PAA HO-P(=O)(OH)-CH₂C(=O)OH	
XIII HO-P(=O)(OH)—CH(CH₃)-C(=O)OH	
XIV HO-P(=O)(OH)—CH(C₆H₅)-C(=O)OH	
XV HO-S(=O)(=O)-CH₂-C(=O)OH	

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500 μM, only carbonyldiphosphonate (IX) showed a significant inhibition of hepatitis B virus DNA polymerase These results indicate that active inhibitors have two acid groups at close proximity and that at least one should be a phosphono group It seems likely that PFA interferes with a pyro phosphate binding site on the polymerase

The high incidence of hepatitis B virus infections makes the development of efficient chemotherapy important Interferon (for review see 7), virazole (15) and vidarabine (3 25) have been tested in clinical trials The significant but transient effect so far reported for interferon and vidarabine might be improved by longer treatment However, the toxicity of vidarabine (29) is likely to pose restrictions on the length of treatment with this compound and interferon is still available only in limited quantities The viral DNA polymerase activity is not inhibited by vidarabine triphosphate (*Nordenfelt* unpublished observation)

Since PFA has a low cellular (32) and animal toxicity (8) it could be considered as a candidate for clinical trials on hepatitis B PFA inhibits herpes simplex DNA polymerase *in vitro* and has a therapeutic activity in animals infected with herpes virus (1) It is possible that the Dane particle DNA polymerase activity is necessary for hepatitis B virus multiplication and studies on chimpanzees have been initiated to test this assumption (*R Purcell & K Tsiquaye* personal communications)

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PATHOGENESIS OF SENDAI VIRUS INFECTION IN MICE

On the Possible Role of Interferon on the Development of Disease

MIKLOS DEGRÉ and HALVOR ROLLAG Jr

Kapt W Wilhelmsen og Frues Bakteriologiske Institutt University of Oslo Rikshospitalet Oslo Norway

Degre M & Rollag H Pathogenesis of Sendai virus infection in mice On the possible role of interferon in the development of disease Acta path microbiol scand Sect B *89* 177-181 1980

Intraperitoneally (i p) injected interferon prolonged the survival time of mice inoculated intranasally (i n) with Sendai virus and reduced the mortality in mice inoculated i n with *Haemophilus influenzae* Moderate concentrations of interferon were demonstrated in homogenized lungs of Sendai virus infected mice as long as the virus was present Similar concentrations could be produced by i p injection of Sendai virus or interferon Alveolar macrophages from mice treated i p with interferon or Sendai virus phagocytized more actively than control macrophages From the present and earlier data it is concluded that interferon may have a direct effect on the Sendai virus infection The total effect of virus pneumonia is a reduction of the lung macrophage antimicrobial activity and therefore the phagocytosis modifying effect of interferon produced in the lungs is probably of minor importance for the outcome of the disease

Key words Interferon phagocytosis Sendai virus alveolar macrophages

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Parainfluenza I virus Sendai virus is a common …sp…atory tract pathogen in the mouse Since the …aturally transmitted virus usually produces a mild …elf limiting infection it is considered an excellent …model of human parainfluenza virus respiratory …nfection

In addition to the primary virus infection the …sease is frequently complicated by secondary …acterial infections complications which may be …ecisive for the outcome of the disease (9)

The pathogenesis of the viral respiratory infection …s complex involving a number of host defence …actors both specific and non specific Interferon …has long been recognised as a non specific defence …factor against viral infections Evidence for its role in the total resistance has been presented by many authors although such evidence is mainly circum…stancial and in many cases disputable (3)

The present communication is an attempt to analyse the role of interferon in the recovery from murine Sendai virus infection especially the role of the interferon effect on the alveolar macrophage activity

MATERIALS AND METHODS

Mice Inbred young HaM/ICR/CSF/Bom female albino mice were used for all experiments except those requiring

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Virus Parainfluenza I virus Sendai strain was originally obtained from Dr *L A Glasgow* (University of Rochester Rochester N Y) Our strain had been passaged 5 times in embryonated eggs in our laboratory Virus titres were assayed in embryonated eggs and by the haemagglutination (HA) test using guinea pig erythrocy

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In vitro phagocytosis was performed with *Escherichia coli* strain x-7, sero-group 0 86, B 7 (21)

Interferon Murine type I interferon was produced in L-929 cells, inoculated with NDV After incubation overnight at 37 °C the supernatant was removed and clarified by low speed centrifugation The remaining NDV was inactivated by treatment at pH 2 at 4 °C for 4 days Some of the non-interferon proteins were precipitated by this treatment and removed by centrifugation at 1500 *g* for 20 minutes The supernatant was then neutralized to pH 7 2 and used without further concentration or purification Samples of this preparation were kept at −70 °C and fresh ampoules were used for each experiment Interferon activity was assayed by the infectivity inhibition microtest (6) employing L-929 cells and VSV as challenge virus The titres were adjusted to the mouse reference preparation G-002-904-511 obtained from the National Institute of Allergy and Infectious Diseases Bethesda Md The interferon preparation employed in the present study contained 1 × 10^5 units per ml

Phagocytosis experiments Alveolar macrophages were obtained via the trachea (7) The pulmonary lavage was performed with 1 5 and 1 ml of Eagles minimal essential medium (MEM) containing lignocain (16) 20 per cent fetal bovine serum and antibiotics The cells were distributed into Leighton tubes containing »flying coverslips« After incubation overnight at 37 °C in humid atmosphere with 5 per cent CO_2 the non adherent cells were removed by vigorous washing The macrophages were re incubated with the bacterial suspension 10^7 bacteria per ml in Krebs-Ringer-Glucose buffer without serum as described in detail elsewhere (23) The tubes were incubated stationarily at 37 °C After 90 minutes of incubation the »flying coverslips« were removed washed with physiological saline fixed with alcohol and stained with Giemsa The proportion of macrophages containing bacteria and the average number of bacteria per cell were determined microscopically independently by two persons

Statistical analysis LD_{50} was calculated by the method described by *Reed & Muench* Comparison of the mortality rates was made by means of the χ^2 test (employing the 2 × 2 table analysis)

EXPERIMENTS AND RESULTS

In the first experiment we examined the effect of interferon on the course of the Sendai virus infection Mice were inoculated i n with 1 LD_{50} Sendai virus A randomly-selected half of the group of mice (n = 32) were injected intraperitoneally (i p) with 1 × 10^4 units of interferon in 0 1 The injections were started at the same time as virus inoculation and were repeated daily The other half of the group (n = 32) were given the same volume of MEM i p daily The groups were observed for 15 days and the mortality recorded The data are summarized in Fig 1 final mortality was practically the same in interferon-treated as in control mice (68 and 64 5 per cent respectively) However, mice treated with interferon survived longer (mean 9 8 days) than control (mean 8 3 days) Significantly more control died during the first 9 days of observation (... versus 7) and more interferon-treated mice after day 9 (17 versus 4, χ^2 = 8 9, $p < 0 01$)

The concentration of interferon in the ... following the i p injection of 1 × 10^4 units determined Three mice were killed at designated times after injection Their lungs were removed aseptically and homogenized in 1 ml MEM homogenized lungs were centrifuged at 1500

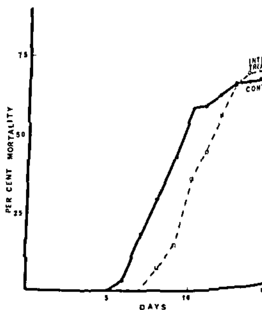


Fig 1 Mortality of mice following inoculation Sendai virus Influence of daily i p injection of 1 units of interferon

1 *Interferon Concentrations in Lung Tissue of ...llowing Intraperitoneal Injection of 1 × 10⁴ Units of Interferon*

...ter injection	Interferon units per ml
...n	170[a]
...n	360
...n	85
...n	10
...n	10

...of three lungs

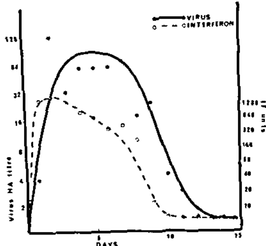


Fig 2 Virus (HA) titres and interferon (IF) units in the lungs of mice following i n inoculation with Sendai virus Each value represent the mean of three lungs

...inutes at room temperature to remove cell ... and the interferon titres were determined in ...pernatants The titres (Table 1) were adjusted ...e dilution factor 1 5 (w/v) of the whole lung ...ml MEM High concentrations of interferon ...present in the lungs during the first hour after ...tion and low titres could be demonstrated for ...ollowing few hours

...e next experiment was designed to correlate ...feron production during the Sendai virus ...tion to the development of the disease A group ...ice were inoculated with 1 LD_{50} of Sendai ...s Three mice were killed daily The lungs were ...

...d 2 high titres persisted to days 7 and 8 and ... declined rapidly after day 8 The interferon ...s correlated well with the virus titres Both ...s and interferon titres declined below detectable ...ls after day 11

...n the following experiment we measured the ...uence of interferon on the phagocytic activity of ...eolar macrophages Mice were injected i p with ... × 10⁴ units of interferon or with 0 1 ml of Sendai virus containing 1024 HA units per ml Eight hours later the mice were killed the macrophages were harvested through pulmonary lavage then seeded into Leighton tubes and their phagocytic activity was determined The lungs of mice inoculated with Sendai virus contained 2–5 × 10^2 units of interferon at the time when the cells were obtained The results shown in Table 2 indicate that macrophages from mice injected with either interferon or Sendai virus phagocytized more actively than macrophages from control mice

To assay the possible role of the enhanced phagocytosis *in vivo* mice were inoculated i n with suspensions of *H influenzae* in concentrations that produced fatal disease in at least 50 per cent of the mice One group of mice (n = 39) were treated daily with 2 5 × 10⁴ units of interferon i p The control mice (n = 42) received daily injections of an

...BLE 2 *Influence of Intraperitoneal Injection of Interferon or Sendai Virus on the Phagocytic Activity of the Alveolar Macrophages*

...reatment	Per cent macrophages containing bacteria	Mean number of bacteria per macrophage
...ontrol	54 7 (54 0–55 1)[a]	0 95 (0 87–1 38)
...nterferon 5 × 10⁴ U	75 0 (73 6–76 4)	2 0 (1 8 –2 16)
...endai virus 100 HA U	76 5 (76 1–87 7)	1 66 (1 59–3 65)

...Median (range) of six counts
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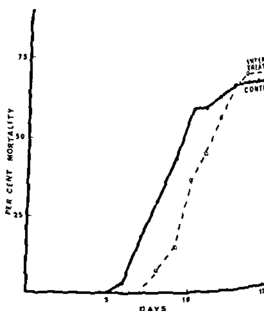


Fig 1 Mortality of mice following inoculation of Sendai virus Influence of daily i p injection of 1 × … units of interferon

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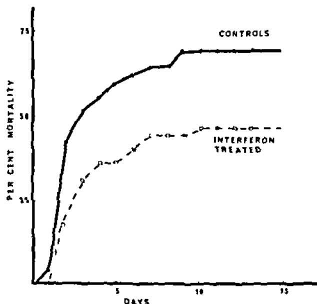


Fig 3 Mortality of mice following inoculation with *Haemophilus influenzae* Influence of daily i p injection of 2 5 × 10⁴ units of interferon

identical volume (0 25 ml) of MEM As shown in Fig 3 the mortality of interferon treated mice was significantly reduced from 68 to 46 per cent (χ^2 = 4 65 p <0 05) However the treatment did not prolong the survival time of those mice that succumbed to the infection The mean numbers of days of survival were 3 9 and 3 6 respectively

DISCUSSION

Being a good model for human respiratory infections the pathogenesis of murine Sendai virus infection has been investigated by several authors There are a number of host defence factors both specific and non specific ones which probably play some part in the termination of the infection The decisive role of the specific B cell mediated humoral immune response has been demonstrated (4 5 15 22) Also the T cell and NK cell mediated local response seems to be of importance (1 2) although it has been questioned whether they are essential for the recovery (15)

Among the non specific defence factors the bactericidal activities of pulmonary macrophages and the physical transport mechanism of the ciliary epithelium of the bronchial system are both deeply involved in the defence against secondary bacterial infections (8 12 14 18 19 20) which are to a large extent responsible for the late mortality following Sendai virus infection of mice (9 10) Interferon is believed to be a defence factor in primary virus infection also in the respiratory tract (15 22) In our experiments parenterally injected interferon prolonged the survival time of Se[...] virus infected mice However these mice alread[...] had interferon in their lungs being induced b[...] virus Thus the significance of the add[...] injected interferon is uncertain

During recent years it has become evident [...] interferon exerts a regulatory effect on a wi[...] of cellular functions (25) including several d[...] factors against infectious diseases Modera[...] centrations of interferon enhance the pha[...] activity of macrophages *in vivo* and *in vitro* (11 [...] 17 24) while high concentrations can depress [...] uptake of bacteria into murine peritoneal macrop[...]ges (24)

Interferon injected parenterally in this stu[...] reduced rather than enhanced the mortality due [...] the inoculated *H influenzae* The interferon conc[...]tration in the lungs following parenteral inje[...] was moderate comparable to that shown [...] enhance phagocytic activity (17 24) It is po[...] that the phagocytosis-enhancing activity can exp[...] the interferon effect on the development of [...] disease but other factors may be involved

The effect of interferon treatment on the Se[...] virus infection is more difficult to explain Du[...] the early phase of the infection *i e* the first 8 [...] while virus was present in the lungs mortality [...] reduced by interferon treatment but the [...] mortality was the same

It has been repeatedly demonstrated that the microbicidal capacity in the lungs and the pha[...]tic activity of macrophages are reduced during [...] infection (8 18 19 20 26) This reductio[...] usually maximal 4–7 days after inoculation o[...] virus The mechanism of this effect is not clear [...] have attempted to find out whether the interf[...] production during infection is involved

During the Sendai virus infection interferon [...] present in the lungs in the early phase as l[...] virus was detectable in the lungs The [...] concentrations of interferon are difficult to [...]lish and it is possible that the local concentrat[...] the alveoles *i e* in the close environment o[...] macrophages was higher than shown by our [...] In *in vitro* studies 5 × 10⁴ units per ml [...] required to depress significantly the phagoc[...] (24) and it is improbable that concentrations o[...] magnitude were present in the alveoles We [...] therefore that the demonstrated effect of inter[...] on the phagocytic activity of the alveolar macro[...]ges is of minor if any importance for the incr[...] susceptibility of virus infected animals to secon[...] bacterial infection The reduction in the phago[...] activity of macrophages can probably be expl[...] by other mechanism(s) *e g* by a direct effe[...] virus on the cells

BRIEF REPORT

TRISODIUM PHOSPHONOFORMATE INHIBITS WOODCHUCK HEPATITIS VIRUS ASSOCIATED DNA POLYMERASE

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Nordenfelt, E & Werner B Trisodium phosphonoformate inhibits woodchuck hepatitis virus associated DNA polymerase Acta path microbiol scand Sect B 88 183–184 1980

Recently a new virus has been described which infects woodchucks *Marmota monax* This virus named woodchuck hepatitis virus (WHV) is closely related to human hepatitis virus (HBV) The virions have the same principal antigenic system involving surface and core determinants and a serological relationship has been found WHV has also a DNA polymerase associated with the core It has previously been reported that trisodium phosphonoformate (PFA) but not phosphonoacetic acid (PAA) inhibits DNA polymerase associated with HBV This investigation shows the same type of inhibition pattern by PFA and PAA on WHV DNA polymerase

Key words Hepatitis B DNA polymerase inhibitor phosphonoformic acid (PFA) woodchuck hepatitis virus

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Recently a new virus has been described which infects woodchucks *Marmota monax* (*Summers et al* 1978) This virus named woodchuck hepatitis virus (WHV) is closely related to human hepatitis B virus (HBV) Infection with either virus results in the accumulation in the blood of excess viral coat protein in the form of 20–25 nm spherical and tubular particles (*Bayer et al* 1968 *Summers et al* 1978) and 40–50 nm diameter particles (*Dane et al* 1970 *Summers et al* 1978) containing DNA (*Robinson et al* 1974 *Summers et al* 1978) and a DNA polymerase (*Kaplan et al* 1973 *Summers et al* 1978)

A serological relationship has been found between viral antigens of HBV and WHV (*Werner et al* 1979) The virions have the same principal antigenic systems involving surface and core determinants The core antigens share major determinants but possess unique antigenic specificities as well The surface antigens of the two vi[illegible]

the [illegible]

any [illegible]

to [illegible] members of a novel class of viruses Like HBV WHV has been shown to be associated with both chronic hepatitis and hepatocellular carcinoma (*Summers et al* 1978) Therefore woodchucks are a possible model for studying various aspects of the pathogenesis of hepatitis B infections

It has previously been reported that trisodium phosphonoformate (PFA) but not phosphonoacetic acid (PAA) inhibits DNA polymerase associated with HBV (*Nordenfelt et al* 1979) Further studies on the mechanism of this inhibition have shown it to be of a non-competitive type and highly specific (*Nordenfelt et al* 1980) We now report on the effect of PFA and PAA on the DNA polymerase associated with WHV

One ml samples of serum specimens for DNA polymerase assay were prepared as described previously (*Nordenfelt & Kjellen* 1975) The sera were taken from woodchucks known to be carriers of WHV Electron microscopy on one of the pellets used in the assay showed a large number of WHV particles Assay conditions were as described previously (*Kaplan et al* 1973) [illegible]

[illegible]

according to the method of *Nylen* (1924) The inhibitors were added to the reaction mixtures at 4 °C prior to incubating for 3h at 37 °C Assays were carried out in duplicate

THE OCCURRENCE OF THE TREHALOSE FERMENTING, TETRACYCLINE AND POLYMYXIN RESISTANT PHENOTYPE AMONG THE *ENTEROBACTERIACEÆ*

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Siboni K The occurrence of the trehalose fermenting tetracycline and polymyxin resistant phenotype among the *Enterobacteriaceæ* Acta path microbiol scand Sect B 88 185–188 1980

In *Proteus morganii P mirabilis* and *Providencia stuartii* the ability to ferment trehalose and resistance to tetracycline were associated in 90%–97% of the strains The same was true of at least 78% of the strains of *Serratia marcescens Proteus vulgaris* showed a more quantitative association of the two traits As the characters occur independently in 3–10% of the strains the association is considered to be due to simultaneous selection in some natural niche The trehalose fermenting tetracycline and polymyxin resistant species ferment few other carbohydrates fewer than the remainder of the *Serratia* species

Key words *Proteus Providencia Serratia* polymyxin resistance tetracycline resistance trehalose fermentation

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A previous paper (*Siboni* 1976) was devoted to the description of the correlation of trehalose fermentation (tr⁺) and resistance to tetracycline (T-R) in *Proteus morganii* It was concluded that *P morganii* could be devided into two natural groups A consisting of tr⁻, T-S strains, and B of tr⁺, T-R strains as the combinations tr⁻T-R and tr⁺,T-S were rare

The present study has concentrated on a description of the occurrence of the tr⁺ T-R phenotype among E…

MATERIALS AND METHODS

The strains were collected from patients specimens in the period 1975–78 Media and sensitivity testing were as described by *Siboni* (1976) For the diffusion method was used the medium described by *Casals & Pedersen* (1978) Fermentation media were read 1–7 days after inoculation and the strains were identified according to *Edwards & Ewing* (1972) and to Grimont & al (1977) The reactions used for identification of swarming strains of *Proteus mirabilis* and *Proteus vulgaris* were production of indol –/+ presence of ornithine decarboxylase +/– fermentation of lactose –/– maltose –/+, and trehalose +/late and liquefaction of gelatin +/+

Tr⁻ mutants of tr⁺ *P morganii* were isolated as described earlier (*Siboni* 1976)

The correlation between results from testing sensitiv

The DNA polymerase activity in the presence of inhibitor as determined by the incorporation of ^{32}P-TTP into acid precipitable radioactivity was compared to the incorporation without inhibitor As shown in Table 1, PFA inhibits WHV associated DNA polymerase activity to approximately 80 % at a concentration of 500 µM and about 60 % at 100 µM This is a clear inhibitory effect but less than the effect on the HBV polymerase which is inhibited to 90 % by 10 µM PAA has less effect The results in these experiments vary with a difference between 0 to 50 % inhibitory effect however, the mean of about 26 % is in complete accordance with the effect on the HBV-associated DNA polymerase

The inhibitory effect of PFA on HBV and WHV associated DNA polymerases appears to be very specific For instance a 50 % inhibition of cellular DNA synthesis has been seen only at a concentration of 2000 µM PFA (*Stenberg & Larsson* 1978) and cellular DNA polymerase γ is not inhibited by 500 µM (*Eriks*... personal communication) No inhibition of bacterial ... mycoplasmal growth by PFA has been observed at 16... µM (unpubl obs) Furthermore the inhibition by PFA but not by PAA discriminates these polymerases f... several other polymerases tested (*Helgstrand et al* 19... *Reno et al* 1978) It has therefore been suggested th... PF... test ... *al* ... polymerase

Additionally it is known that PFA inhibits her... simplex DNA polymerase *in vitro* and has a therape... activity in animals infected with herpes virus (*Alen*... *al* 1978) If the HBV and WHV DNA polymerases ... necessary for viral replication it is possible that inhibiting them PFA could have a therapeutic eff... Studies have been initiated to test this hypothesis

TABLE 1 *Inhibition of WHV DNA Polymerase Activity by PFA and PAA in Serumpreparation from 4 Woodchu...*

Animal	Incorporation in cpm in the absence of PFA or PAA	500 µM PFA percentage inhib	100 µM PFA percentage inhib	10 µ PFA percentage inhib	500 µM PA... percentage inh...
1	234		47	26	
	1920	79	56		0
2	42526	88	73	40	
3	20344	89			57
4	10281		71	28	21

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Acta path microbiol scand Sect B 88 185–188 1980

THE OCCURRENCE OF THE TREHALOSE FERMENTING, TETRACYCLINE AND POLYMYXIN RESISTANT PHENOTYPE AMONG THE *ENTEROBACTERIACEÆ*

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Key words *Proteus Providencia Serratia* polymyxin resistance tetracycline resistance trehalose fermentation

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A previous paper (*Siboni* 1976) was devoted to the description of the correlation of trehalose fermentation (tr⁺) and resistance to tetracycline (T R) in *Proteus morganii* It was concluded that *P morganii* could be devided into two natural groups A consisting of tr⁻ T S strains and B of tr⁺ T R strains as the combinations tr T R and tr⁺ T S were rare

The present study has concentrated on a description of the occurrence of the tr⁺ T R phenotype among *Enterobacteriaceæ* naturally resistant to polymyxin namely other *Proteus* species *Providencia* and *Serratia* It will de discussed how tr⁺ T R species differ from [illegible] T-S and tr⁻ T R species

MATERIALS AND METHODS

The strains were collected from patients specimens in the period 1975–78 Media and sensitivity testing were as described by *Siboni* (1976) For the diffusion method was used the medium described by *Casals & Pedersen* (1978) Fermentation media were read 1–7 days after inoculation and the strains were identified according to *Edwards & F*[illegible] [illegible] tion of indol –/+ presence of ornithine decarboxylase +/– fermentation of lactose –/– maltose –/+ and trehalose +/late and liquefaction of gelatin +/+

Tr⁻ mutants of tr⁺ *P morganii* were isolated as described earlier (*Siboni* 1976)

The correlation between results from testing sensiti

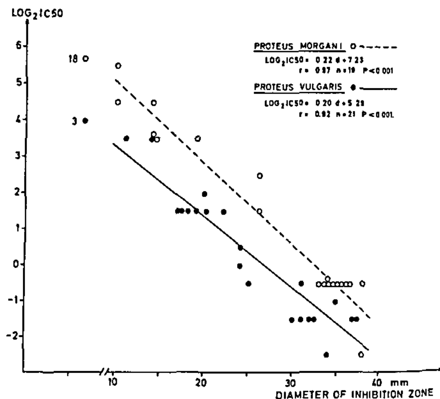


Fig 1 Sensitivity to tetracycline of selected strains of *P morganii* and *P vulgaris* IC50 is correlated to the diame the inhibition zone The same inhibition zone corresponds to a lower IC50 with *P vulgaris* the difference average of 1 31 $\log_2$ (2 48 times)

vity to tetracycline of strains of *P morganii* and *P vulgaris* by the diffusion method and by the tube dilution method is shown in Fig 1

The strains of *Serratia* and their sensitivity to tetracycline have been described elsewhere (*Siboni* 1980)

RESULTS

P morganii To the strains collected in 1975 (*Siboni* 1976) have been added 131 strains from 1976 The distribution (Table 1) is still incompatible with a

TABLE 1 *Strains of* Proteus morganii *Distributed According to Fermentation of Trehalose and Sensitivity to Tetracycline*

	Trehalose fermenter	Trehalose non fermenter	Totals
Tetracycline resistant	18	21	39
Tetracycline sensitive	2	182	184
Totals	20	203	223

TABLE 2 *Strains of* Proteus mirabilis *Distr According to Fermentation of Trehalose and Sens Tetracycline*

	Trehalose fermenter	Trehalose non fermenter	T
Tetracycline resistant	249	5	
Tetracycline sensitive	2	0	
Totals	251	5	

random distribution according to the two c tr^+/tr and T R/T S ($P < 10^{-18}$) *Fisher* and the association tr^+ T R or tr T S exist in 0 90 of the strains

Table 1 shows that tr^+ T S strains do exist these strains tr T S mutants could be isolated

Eight of the 39 T R strains were resista chloramphenicol

P mirabilis The distribution in Table compatible with a random distribution accord the two criteria (P) *i e*

LE 3 *Strains of* Proteus vulgaris *Distributed According to Fermentation of Trehalose and Sensitivity to Tetracycline*

Sensitivity to tetracycline (diameter of inhibition zone)	Number of days till fermentation of trehalose							
	1	2	3	4	5	6	7	Totals
≤ 14 mm	6	1	2	–	–	–	–	9
15 22 mm	6	22	2	–	1	1	–	32
23 27 mm	2	1	3	1	1	–	–	8
≥ 28 mm	–	5	11	5	2	1	1	25
Totals	14	29	18	6	4	2	1	74

Correlation coefficient, r 0 5308 e_r = 0 100 t = 5 3 P < 0 001

rmentation of trehalose was associated with istance to tetracycline in $\frac{249}{256}$ = 0 97 of the ains

P vulgaris There was not the qualitative fference between the tr⁺/tr⁻ and T R/T S strains served with *P morganii* and *P mirabilis* All the strains fermented trehalose sooner or later but e quicker the fermentation the more resistant to tracycline were the strains The figures in Table 3 ve a correlation coefficient r of 0 53 between the umber of days till fermentation of trehalose and e diameter of the inhibition zone with tetracycline

P rettgeri All 17 strains were tr⁻ and T R so the vo characters were not associated

Providencia stuartii All strains were tr⁺ 22 ere T R so the two characters were associated in = 0 92

17 of the strains were resistant to chloramphenicol.

Serratia marcescens All 56 strains were tr⁺ 51 ere T R so the characters were associated in $\frac{51}{56}$ = 0 91 Even if the 33 multiresistant strains with suspected R factors (*Siboni* 1980) are excluded the characters are associated in $\frac{18}{23}$ = 0 78

S liquefaciens The 24 strains were tr⁺ and T S so the two characters were not associated The same was true of *S plymouthica (S kiliensis)* 6 strains and with *S marinorubra (S rubidæa)* 10 strains

All strains of the nine species were resistant to polymyxin B 10 µg/ml with the exception of 4 strains of *P morganii* 3 strains of *P mirabilis* 5 strains of *S plymouthica* and 2 strains of *S marinorubra*

DISCUSSION

Four species *P morganii P mirabilis Providencia stuartii* and *S marcescens* showed association of fermentation of trehalose and resistance to tetracycline in (78) 90–97 % of the strains The few strains which were tr⁺ T S or tr⁻ T R show that the two characters are not dependent on one another This is also shown by the fact that tr⁻ mutants could be selected from tr⁺ strains of *P morganii* without changing the resistance to tetracycline (*Siboni* 1976)

The association of the two characters is therefore considered to be caused by simultaneous selection in a niche where the strains may ferment trehalose if they are resistant to tetracycline e g a niche inhabited by *Streptomyces* species which are able to synthesize trehalose (*Elbein* 1967) and produce tetracycline and chloramphenicol This could explain why so many strains of *Providencia stuartii* and of the T R *P morganii* are resistant to chloramphenicol As chloramphenicol inhibits tetracycline inactivating enzymes (*Hofemeister* 1970) chloramphenicol resistance in a strain would add to its resistance to tetracycline in the presence of both tetracycline and chloramphenicol

This hypothesis is supported by the large tr⁻T-S and chloramphenicol sensitive group of *P morganii* (*Siboni*'s group A 1976) and by the rarity of the tr⁺ T S and tr⁻ T R *strains of this species* When one of the characters has been lost selection is against the other one *P vulgaris also demonstrated* the correlation of trehalose fermentation to resistance to tetracycline (Table 3) but quantitatively as

with the species mentioned above, the correlation was not absolute the correlation coefficient was low, $r = 0.53$, but definitely $\neq O$ ($e_r = 0.01$, $t = 5.3$, $f = 72$, $P < 0.001$)

The correlation tr⁺/T-R was not found in *S liquefaciens S plymouthica* and *S marinorubra* The explanation may be that these species are not so restricted in their carbohydrate sources as are *P morganii P vulgaris P mirabilis Providencia stuartii* and *Serratia marcescens* While the former species ferment five or six dihexoses and monopentoses, the latter ferment less than four

The grouping of *S marcescens* with regard to the traits discussed tr⁺, T-R and polymyxin resistant, with *Providencia stuartii P mirabilis P vulgaris and the tr⁺ group of P morganii* (Siboni s group B 1976) is not so surprising Its DNA is only slightly related to that of *Proteus* (*Brenner et al* 1978) but its overall similarity to *Proteus* is at the 75% level (*Johnson et al 1975) i e at the same level as to S liquefaciens* and to *S plymouthica* and higher than the similarity to *S marinorubra* 70% (*Grimont et al* 1977) The species mentioned have not been compared *in one single investigation*

The niche hypothesis formulated above and to some extent substantiated does not cover the tr⁻ T-R *Proteus rettgeri* Examination of sewage and soil has not so far disclosed the niche suited for the trehalose fermenting, tetracycline and polymyxin resistant species

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SENSITIVITY OF *SERRATIA* TO TETRACYCLINE

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Siboni K. Sensitivity of *Serratia* to tetracycline Acta path microbiol scand Sect B 88 189–192 1980

S marcescens had two levels of resistance to tetracycline bacteriostatic end points 32 and 180 µg/ml the latter group consisted of strains resistant to carbenicillin and to streptomycin *S plymouthica* *S liquefaciens* and *S marinorubra* were sensitive to tetracycline with the last named as the least sensitive species Less difference was found between the bactericidal end points of the four species but there was still 1–2 two fold steps between *S marcescens* and the remaining three species

Key words *Serratia* tetracycline sensitivity

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Different investigators have found 76–86 per cent of strains of *Serratia marcescens* resistant to tetracycline (*Wilfert et al* 1970 85% had MIC > 5 µg/ml *Greenup & Blazevic* 1971 76% had MIC > 25 µg/ml *Grimont et al* 1977 84% had MIC > 25 µg/ml 98% had MIC > 5 µg/ml) *Medeiros & O Brien (1969)* found two levels of resistance to tetracycline MIC – 500 µg/ml and MIC ~ 31–62 µg/ml the strains in the former group were also resistant to sulphonamide streptomycin and chloramphenicol and they were highly resistant to ampicillin MIC ≥ 250 µg/ml these resistance traits were transferable to *E coli* *Farrar & O Dell* (1976) examined a few strains and showed that the multiresistant strains with high ampicillin resistance were resistant to carbenicillin (MIC > 300 µg/ml) while the oligoresistant strains of low ampicillin and tetracycline resistance were sensitive to carbenicillin (MIC ≤ 6 µg/ml)

Greenup & Blazevic (1971) found 15 of their 17 *Serratia liquefaciens* strains sensitive to tetracycline 12 5 µg/ml all the strains were sensitive to streptomycin to chloramphenicol and to carbenicillin Twenty two strains of *Serratia marinorubra* were resistant to tetracycline 5 µg/ml sensitive to 25 [illegible] 977) The same investigators found 20 strains of *Serratia plymouthica* sensitive to tetracycline 5 µg/ml

It has been the purpose of the present investigation to describe the occurrence of *Serratia* in patients specimens the level of resistance to tetracycline in the different species and the correlation of this resistance to other resistance traits

MATERIALS AND METHODS

The strains of *S marcescens* and *S liquefaciens* were isolated from 77 100 specimens received in 1976 and 1977 in the Regional Laboratory of Statens Seruminstitut at Odense University Hospital from hospitals and general practitioners in the county of Funen which has 500 000 inhabitants The strains were isolated on modified Conradi Drigalski agar and identified in

been used

Only three strains of *S marinorubra (S rubidaea)* and one of *S plymouthica (S kiliensis)* were isolated in 1976 and 1977

In the study were therefore included *S marinorubra* Four strains isolated earlier and three strains received from Dr *Hans Lautrop* Statens Seruminstitut

Copenhagen *S plymouthica* Five strains received from Dr *Hans Lautrop* Sensitivity tests Routine diffusion tests were performed using Rosco s tablets (neosensitabs® Rosco DK 2630 Taastrup) on Danish blood agar Statens Seruminstitut as described by *Casals & Pedersen* (1978) Strains showing inhibition zone diameters > 23 mm corresponding to MIC <5 μg/ml tetracycline <20 μg/ml streptomycin <15 μg/ml chloramphenicol <15 μg/ml sulphonamide and <10 μg/ml carbenicillin were regarded as sensitive Determination of IC50 in two fold tube dilution tests and other media were as described by *Siboni* (1976) $IC50 \times \sqrt{2} = MIC$

The resistance types of *S marcescens* determined in diffusion tests are recorded in Table 1 At least four strains of each of the resistance types carbenicillin resistant/tetracycline resistant carbenicillin sensitive/tetracycline resistant and carbenicillin sensitive/tetracycline sensitive of *S marcescens* and of each of the remaining three *Serratia* species were tested by the tube dilution method (Table 2)

The bactericidal concentration was determined as the lowest concentration which killed 99% of the bacterial cells after 18 h of incubation of the broth dilution series This definition was feasible with the small inoculum used average $10^{4\,4}$ cfu (*Barry & Sabath* 1974)

Wilcoxon s non parametric test was used to compare the end points of the different species and groups (*Wilcoxon* 1945)

RESULTS

S marcescens (56 strains) Table 1 shows that strains resistant to carbenicillin were also resistant to tetracycline and to streptomycin and some of them to chloramphenicol while these resistance traits were rarer among the strains sensitive to carbenicillin, none of the latter strains was resistant to chloramphenicol The bacteriostatic end points with tetracycline were higher in the group resistant to carbenicillin than in the carbenicillin sensitive group ($2\alpha < 0\ 01$) (Table 2) The bactericidal end points of the two groups differed less 90 5 – 25 μg/ml ($2\alpha < 0\ 02$)

Five strains of the carbenicillin sensitive group were sensitive to tetracycline in routine diffusion tests Tube dilution tests with these strains gave $IC50 \leq 2^{2\,5} = 5\ 7$ μg/ml (Table 2)

S liquefaciens (24 strains) and *S plymouthica* strains) All strains were sensitive to tetracycline diffusion tests and ten and four strains respectively in tube dilution tests (bacteriostatic end points) *plymouthica* was even more sensitive than *liquefaciens* (Table 2)

S marinorubra (10 strains) was sensitive tetracycline in diffusion tests but the tube dilution test gave a borderline value $IC50 = 2^{2\,55} =$ μg/ml in agreement with *Grimont et al* (19 (Table 2)

The bactericidal end points of the last th species were the same (Table 2)

DISCUSSION

Bacteriostatic end points *S marcescens* had levels of resistance to tetracycline IC50 32 and μg/ml The high level resistance was alm exclusively found in strains resistant to carbenic (Table 2) Table 1 shows that this group consist

TABLE 1 *Strains of* Serratia marcescens *Distributed According to Carbenicillin Resistance and to Other Res* *Types (Diffusion Method)*

Resistance type	Carbenicillin resistant		Carbenicillin sensitive		Totals
	observed	expected	observed	expected	
SuTSC	7	0 70	–	1 25	7
SuTS	13	4 85	8	8 75	21
TS	–		5	9 32	5
SuT	–	14 45	1	6 97	1
T	–		17	6 49	17
O	–		5	3 21	5
Totals	20	20 00	36	35 99	56

Su T S and C resistant to sulphonamide tetracycline streptomycin and chloramphenical
O sensitive to the named antimicrobials
The expected values have been calculated from products of the frequencies of the single resistance traits expected if the traits occurred independently
(Example $p(SuTSC) = 29/56 \times 51/56 \times 33/56 \times 7/56 = 0\ 035$)

TABLE 2 *Sensitivity to Tetracycline of Selected Strains of* Serratia *with the Tube Dilution Method*

log_2 IC50	−1 5	−1 0	−0 5	0 0	0 5	1 0	1 5	2 0	2 5	3 0	3 5	4 0	4 5	5 0	5 5	6 0	6 5	7 0	7 5	8 0	log_2 geometric mean
IC50 μg/ml		0 5		1 0		2 0	2 8	4 0	5 7	8 0		16 0		32 0		64 0		128		256	
S marcescens																					
carbenicillin R																					
tetracycline R																					
bacteriostatic	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	1	1	10	–	7 38
bactericidal	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	11	1	7 54
carbenicillin S																					
tetracycline R																					
bacteriostatic	–	–	–	–	–	–	–	–	–	–	–	–	7	1	4	–	2	–	1	–	5 27
bactericidal	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	5	4	5	1	7 07
carbenicillin S																					
tetracycline S																					
bacteriostatic	–	–	–	–	1	–	1	–	2	–	–	–	–	–	–	–	–	–	–	–	1 75
bactericidal	–	–	–	–	–	–	–	–	–	–	1	–	–	–	–	–	–	–	3	–	6 50
S liquefaciens																					
bacteriostatic	–	–	–	–	6	–	4	–	–	–	–	–	–	–	–	–	–	–	–	–	0 90
bactericidal	–	–	–	–	–	–	–	–	–	–	–	–	–	2	3	1	3	1	–	–	5 90
S plymouthica																					
bacteriostatic	2	–	2	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	−1 00
bactericidal	–	–	–	–	–	–	–	–	–	–	–	–	–	–	2	2	–	–	–	–	5 75
S marinorubra																					
bacteriostatic	–	–	–	–	–	–	–	1	8	–	1	–	–	–	–	–	–	–	–	–	2 55
bactericidal	–	–	–	–	–	–	–	–	–	–	–	–	1	1	1	–	4	–	3	–	6 35

R resistant and S sensitive with the diffusion method

multiresistant strains in much higher numbers than could be expected if the resistance traits occurred independently ($P < 10^{-5}$) (Table 1 column expected) This agrees with the findings of *Medeiros & O Brien* (1968) *Farrar & O Dell* (1976) and *Søgaard* (1979) for *E coli* The carbenicillin sensitive group was dominated by strains resistant only to tetracycline and mainly of low level resistance (Table 2)

The two levels of resistance to tetracycline are implicit in the results of *Greenup & Blazevic* (1971)

The remaining three species were sensitive to tetracycline with *S marinorubra* as the most resistant but they were more sensitive than the low level tetracycline resistant group of *S marcescens* ($2\alpha < 0\ 01$) Bactericidal end points The difference between the species was less than with the bacteriostatic end points amounting at most to fourfold The end points of the carbenicillin sensitive group of *S marcescens* were lower than those of the carbenicillin resistant group ($2\alpha < 0\ 02$) and the end points of the three remaining species were lower than those of *S marcescens* as a whole ($2\alpha < 0\ 01$)

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ANTIBIOTIC SENSITIVITY OF *HAEMOPHILUS INFLUENZAE* STRAINS INCLUDING THREE RECENT CHLORAMPHENICOL-RESISTANT ISOLATES

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Zackrisson, G & Brorson J E Antibiotic sensitivity of *Haemophilus influenzae* strains including three recent chloramphenicol resistant isolates Acta path microbiol scand Sect. B 88 193–198 1980

The antibiotic sensitivity of 100 recent isolates of *Haemophilus influenzae* was determined Three strains were resistant to chloramphenicol with minimal inhibitory concentrations of 16 µg/ml Of these three resistant strains one produced betalactamase and one was resistant to sulfamethoxazole trimethoprim The remaining strains were inhibited by 0 25–2 0 µg/ml of chloramphenicol Ampicillin and benzylpenicillin were found to inhibit all but the betalactamase producing strains at low concentrations Regarding sulfamethoxazole trimethoprim 96% had minimal inhibitory concentrations of 2 5–0 12 µg/ml or less while two strains were resistant The invitro efficacy of erythromycin against *H influenzae* was low The majority of the strains was inhibited by low concentrations of doxycycline and cefuroxime while cefoxitin exhibited minimal inhibitory concentrations values usually exceeding 1 µg/ml The minimal inhibitory concentrations registered are compared to the concentrations of the different antibiotics attainable in certain body fluids

Key words *Haemophilus influenzae* antibiotic sensitivity chloramphenicol resistance

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There are some previous reports on chloramphenicol resistant strains of *H influenzae* with minimal inhibitory concentrations (MICs) varying from 8 to 50 µg/ml (4 7 10 14 20 24 25 29 32 39) In this laboratory we have recently isolated three strains of *H influenzae* resistant to chloramphenicol and to the best of our knowledge we are not aware of previous Scandinavian reports on chloramphenicol resistant *H influenzae* strains

These facts prompted us to test the susceptibility of 100 recent clinical isolates of *H influenzae* to the antibiotics most frequently used when treating *H influenzae* infections

MATERIALS AND METHODS

Bacterial strains The 100 strains of *H influenzae* tested were all recent clinical isolates Three strains originated from blood cultures 5 from conjunctival secretions 3 from middle ear exudates 1 from maxillarly sinus 1 from wound and 87 from patients with upper respiratory tract diseases Only one strain from each patient was tested The strains were identified by culture characteristics and growth dependence of X and V factors The organisms were stored at –26 °C for three months to 1 week in nutrient broth containing horse serum and glucose

Media The antibiotic susceptibily tests were performed as broth microdilution tests described by *Kirven & Thornsberry* (21) Mueller Hinton broth pH 7 0 (Oxoid CM 405) was supplemented with 0 2% Hemin (H896 Bovin/Equin Sigma) and 0 5% nicotinamideadenine dinucleotide (Sigma D 7381) (M H broth) The MIC 2000 dispenser (Dynatech Laboratories Alexandria Va USA) was used for dispensing 100 µl amounts of broth containing antibiotics into the wells of microdilution plates The plates were stored at –26 °C for a maximum period of 3 weeks prior to use The antibiotic concentrations of the solutions dispensed were checked both by conventional microbiological assays (11) and by observing the MICs of bacterial strains with known

susceptibility *E coli* ATCC 25922, *Staphylococcus aureua* ATCC 25923 and *Streptococcus faecalis* ATCC 29212 The chloramphenicol MICs of the resistant strains were performed in tubes containing 1 ml of broth

Antibiotics All antibiotics were of known potency and were used in twofold dilutions

Determination of Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) The strains were cultured in supplemented M H broth for 24 hours and were diluted to contain about 5 10^7 CFU/ml The microdilution plates were inoculated with the Dynatech MIC 2000 inoculator and each well received approximately 10^4 CFU The microdilution plates were incubated for 18 hours at 36 °C in 5% CO_2-atmosphere The MICs were read as the lowest concentration of drugs that gave no visible growth For MBC determinations the Dynatech inoculator was used for transferring inoculum from each well to chocolate agar plates The MBCs were read after 24 hours incubation at 36 °C in 5% CO_2 atmosphere as the lowest concentration giving no growth

Beta-lactamase test Beta lactamase production was detected by the chromogenic cephalosporin (30)

Enzyme production Inactivation of chloramphenicol by the chloramphenicol resistant strains was demonstrated by the technique of *Slack et al* (36) A chocolate agar plate was seeded with a chloramphenicol sensitive *E coli* Colonies from the chloramphenicol resistant *H influenzae* strains were applied to filterpaper discs, one disc for each strain A chloramphenicol sensitive *H influenzae* strain used as control was applied to the surface of two other filterpaper discs After about five minutes a 30 µg chloramphenicol disc was placed on the top of each disc except on one of the discs containing the sensitive *H influenzae* strain The plate was read after incubation for 18 hours at 37 °C

RESULTS

The results of the susceptibility tests are presented in Fig 1 A–H and Table 1

Fig 1 MIC —— and MBC - - of 100 strains o *Haemophilus influenzae* expressed as cumulative percen tages of strains inhibited with increasing drug concentra tion (A) erythromycin, (B) ampicillin (C) doxycycline (D) chloramphenicol (E)cefuroxime (F) cefoxitin (G sulfamethoxazole trimethoprim (H) benzylpenicillin

Erythromycin The MICs of 38% of the strain exceeded 5 µg/ml The remaining strains wer inhibited by concentrations from 1 25 to 5 0 µg/n with median MIC 4 37 µg/ml MBC was >5 µg ml for 68% and 2 5–5 0 µg/ml for the remainin strains

Ampicillin Two strains were betalactamase pro ducing with MICs more than 5 µg/ml Th majority of the strains had MICs between 0 31 an 0 62 µg/ml with median MIC of 0 34 µg/m MBCs were identical to the MICs for all except strains that had MBCs one twofold concentratio higher

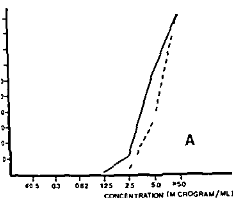
Doxycycline The MICs of 35% of the strai were less than 0 31 µg/ml while 63 had MICs fro 0 62 to 2 5 µg/ml Two strains were resistant wi MICs 5 0 and 10 0 µg/ml Almost all strains h MBCs a dilution higher than their MICs

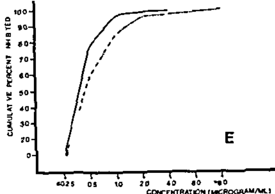
Chloramphenicol All strains except the resistant isolates were inhibited by 0 25–2 0 µg/n mean 0 53 µg/ml, MBCs for the 97 sensiti strains were 0 5–4 0 µg/ml, mean 1 1 µg/ml

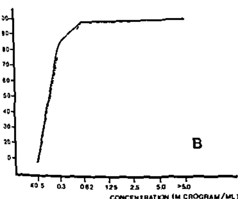
Figure 2 demonstrates a zone of inhibiti around the disc soaked with the sensitive strain b not around the filterpaper discs containing t resistant strains MICs of the chloramphenic resistant strains are presented in Table 1 The fi isolate (No 1) was a *H influenzae* type b deriv from the nasopharyngeal secretion of a 7 year o boy, earlier treated with penicillin for repeat episodes of otitis media

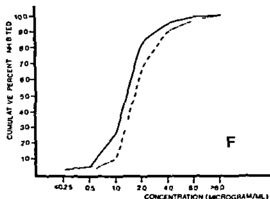
TABLE 1 *Antibiotic Sensitivity of the Chloramphenicol Resistant Strains*

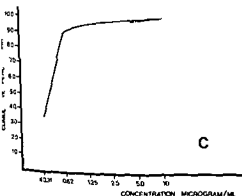
Strain	MIC µg/ml			MBC µg/ml		
	1	2	3	1	2	3
Erythromycin	5 0	>5 0	5 0	>5 0	>5 0	>5 0
Ampicillin	<0 15	>5 0	0 31	<0 15	>5 0	0 31
Doxycycline	1 25	1 25	2 5	1 25	1 25	2 5
Chloramphenicol	16 0	16 0	16 0	46 0	23 0	23 0
Cefuroxime	<0 25	0 5	0 5	<0 25	0 5	0 5
Cefoxitin	1 0	2 0	2 0	2 0	2 0	2 0
Sulfamethoxazole/ Trimethoprim	>2 5 /0 12	2 5 /0 12	>80 0 /4 0	80 0 /4 0	40 0 /2 0	>80 0 /4 0
Benzylpenicillin	<0 25	>8 0	0 5	<0 25	>8 0	0 5
Betalactamase	-	+	-	-	+	-

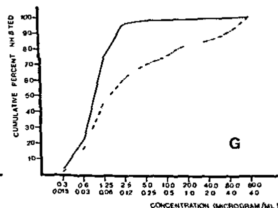

A
CONCENTRATION (MICROGRAM/ML)

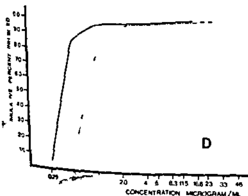

CUMULATIVE PERCENT INHIBITED
E
CONCENTRATION (MICROGRAM/ML)

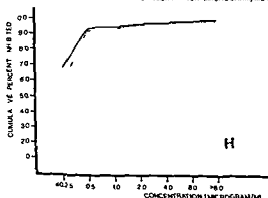

B
CONCENTRATION (MICROGRAM/ML)


CUMULATIVE PERCENT INHIBITED
F
CONCENTRATION (MICROGRAM/ML)


C
CONCENTRATION MICROGRAM/ML


CUMULATIVE PERCENT INHIBITED
G
CONCENTRATION (MICROGRAM/ML)


D
CONCENTRATION MICROGRAM/ML


CUMULATIVE PERCENT INHIBITED
H
CONCENTRATION (MICROGRAM/ML)

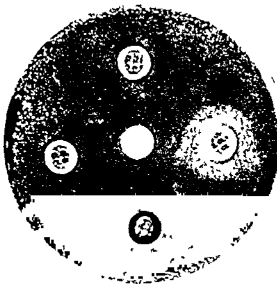


Fig 2 Demonstration of chloramphenicol inactivation In the middle Control sensitive strain (no chloramphenicol disk applied) To the right Chloramphenicol-sensitive strain The remaining disks The three chloramphenicol inactivating strains

The second chloramphenicol-resistant strain (No 2) was isolated from the epipharyngeal culture of a 1-year old girl with recurrent otitis media, treated in the past with penicillin This strain produced betalactamase and was nontypable

The third chloramphenicol-resistant strain (No 3) was nontypable and was isolated from a chronic neck fistula of a 18-year old woman The strain was also resistant to sulfamethoxazole trimethoprim Two months earlier exudate from the fistula had grown a *H influenzae* strain sensitive to both SXT and chloramphenicol

The three chloramphenicol-resistant strains had moderately reduced sensitivity to doxycycline

Cefuroxime The majority of the strains had MICs of 0 5 μg/ml or less while 3% were less sensitive with MICs of 2 0–4 0 μg/ml The betalactamase producing strains had both MICs and MBCs of 0 5 μg/ml for cefuroxime

Cefoxitin Fig 1 F demonstrated that three of the strains were inhibited by less than 0 25 μg/ml One strain had MIC higher than 8 0 μg/ml The remainder had MICs from 0 5 to 8 0 μg/ml median value 1 93 μg/ml The MBCs were mostly identical to the MICs A few had MBCs a dilution step higher than their Mics

Sulfamethoxazole-Trimethoprim The MICs of 96% of the strains were 2 5/0 12 μg/ml or less while 2 strains had MICs exceeding 80 0/4 0 μg/ml Despite low MICs several strains demonstrated high MBCs

Benzylpenicillin Four strains had MICs of 1 0–4 0 μg/ml and two were betalactamase-producing while the majority were inactivated by 0 5 μg/ml o less The MBCs were mostly identical to the MIC

DISCUSSION

The recent observations that certain strains of *H influenzae* have acquired resistance not only ampicillin but also to chloramphenicol is of concern when treating serious infections caused b *H influenzae*

In the present study three strains with decreas susceptibility to chloramphenicol were registere These strains were found to inactivate chloramph nicol probably as a result of enzymatic activity accordance with other studies on chloramphenic resistant *H influenzae* strains (22, 25) *van Kling ren et al* have demonstrated that the chloramphe col resistance of *H influenzae* is plasmid mediat and probably transferred by conjugation (22)

The results registered in this study apart from t chloramphenicol-resistant strains are well in acco dance with previous reports on the susceptibility *H influenzae* to chloramphenicol (12, 14 40 4 In the present study 98% of the strains had l MICs when tested against SXT This is in sha contrast to the figures presented by *May & Davi* who demonstrated 55% resistance to SXT empl ing a disk diffusion method (26) A recent rep demonstrates a correlation between *H influen* strains with high MBCs to SXT and p eradication of strains with this ability from nasopharynx of children (21, 42) In the pres study a considerable portion of the strains w shown to have a high MBC despite a low MIC

It has recently been demonstrated that ventricu concentration of chloramphenicol after doses of mg/kilo bodyweight every 6 hour in newbo children may be unpredictable and so low as jeopardize treatment of *H influenzae* strains w MICs of 4–8 μg/ml (43) This is a most lik explanation for treatment failures reported children However, the doses used in children abo 1 month old and adults ought to result in suffici cerebrospinal concentrations for chloramphenic sensitive strains (38)

Regarding ampicillin all strains in the pres study with the exception of the betalactama producing strains were inhibited by 0 62 μg/ These findings are well in accordance with earl reports (4, 14, 19) Comparison between t concentrations measured in cerebrospinal fluid a ear exudates indicate that ampicillin levels in th body fluids are well above the MICs of no betalactamase-producing strains of *H influenzae* 16, 31 38) Sufficient l

ined in sinus mucosa when treating sinusitis

enetration of both sulfamethoxazole and trime
prim to cerebrospinal fluid and other body fluid is
d (1 13, 34 37) Thus a combination of these
gs could be an alternative in treating *H*
uen.ae-meningitis and other serious infections
sed by this organism Clinical experience regar
g treatment of meningitis is limited but the
nbination has been used with success in certain
es (34) The finding that 2% of the strains had
Cs in excess of levels obtained in CFS is of some
cern.

The sensitivity to cefuroxime and cefoxitin was in
eement with earlier findings (5 27) There are a
reports on cefuroxime treatment of meningitis
33) *Renlund & Pettay* demonstrated concentra
ns of cefuroxime in cerebrospinal fluid from 2 3–
μg/ml in patients with inflamed meninges (33)

furoxime (28)

About one third of the strains had MICs
ceeding 5 μg/ml when tested against erythromy
n. There are conflicting reports on the efficiency of
ythromycin against *H influenzae* *Kirven & Thornsberry* (21) and *Kalm et al* (18) found the
majority of the *H influen.ae* strains to be inhibited

inhibited by 0 5 μg/ml or less *Sell* reported that the
majority of typable and nontypable *H influenzae*
strains were inhibited by 3 12 μg/ml while the type
strains were more sensitive to erythromycin (35)
These differences might be explained by the fact that
the activity of erythromycin is affected by changes
in pH Further the pH-dependence of erythromycin
makes evaluation of concentrations in body fluids
difficult since the pH in certain purulent inflamma
tion processes deviates from normal as demonstra
ted by *Carenfelt & Lundberg* (8) *Bass et al* found
that the concentrations of erythromycin succinate in
middle ear fluid were 0 07–1 02 μg/ml which in
view of our observations seem to be unsatisfactory
levels (6) Further in treatment of otitis media
caused by *H influenzae* *Howie & Ploussard*
observed treatment failure when only erythromycin
was used (15) *Kalm et al* demonstrated concentra
tions of erythromycin stearate in sinus secretion
ranging from 0 3 to 2 5 μg/ml when the orally
given dose was 500 mg 3 times a day while the
measured concentrations in sinus secretion were
0 2–1 1 μg/ml when 500 mg twice a day was
administered The clinical results were better with
the higher dose but the gastrointestinal effects were
more common (18)

The results regarding doxycycline are well in accordance with earlier reports (4, 21) The majority of the strains tested had MICs below the concentra tions of doxycycline demonstrated in sinus secretions and in different tissues including lungs and pleura (3 23)

Benzylpenicillin was highly active in vitro against *H influenzae* strains Our results differ slightly from those reported by *Kamme* (19) and *Barret et al* (4) using agar dilution method The efficacy in vivo in certain cases of pneumonia caused by *H influenzae* was demonstrated by *Asmar et al* treating children with benzylpenicillin (2)

It is becoming increasingly obvious that the treatment of severe *H influenzae* infections has to be guided by proper sensitivity testing as the sensitivity pattern of some strains is unpredictable

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CHANGES IN VIRULENCE, M PROTEIN AND IgG Fc RECEPTOR ACTIVITY IN A TYPE 12 GROUP A STREPTOCOCCAL STRAIN DURING MOUSE PASSAGES

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Burova, L. A Christensen, P Grubb R. Jonsson A Samuelsson, G Schalen, C & Svensson, M L. Changes in virulence M protein and IgG Fc receptor activity in a type 12 group A streptococcal strain during mouse passages Acta path microbiol scand Sect. B 88 199–205 1980

A type 12 group A strain (1800) was passaged serially through mice 25 times The ability to survive in normal human blood dropped from a growth index of 52 after the first passage to 1 after four passages After 14 passages the growth index increased again and stabilized above 30 The virulence for mice increased from a LD_{100} of 10^8 colony forming units (CFU) to 10–100 CFU after 7 passages and then remained constant. The M12 antigen disappeared after 4 passages as tested by immunodiffusion electro immunoassay and indirect bactericidal tests Three antisera raised in rabbits against strains originally belonging to types M3 M12 and M46 but devoid of type antigens after mouse passages showed high bactericidal ind ces against the 1800 strain after 14 or more passages on mice Anti type M1 serum was also found bactericidal for the passaged strains The IgG Fc receptor activity of the strain isolated after each mouse passage was tested in hemagglutination experiments with human red blood cells coated with »incomplete« anti Rh and hot hydrochloric acid extracts of the strains The capacity to agglutinate »Ripley«-coated cells increased gradually during the first 12 passages and subsequently the titres of the extracts stabilized between 1 160 and 1 320 The HUN coat useful for detection of the G3m (5) marker gave titres increasing with the number of passages while the titres for IgG1 coats kept at 1 4 or below On background of these results the possible role of the IgG Fc-receptor as a virulence factor is discussed

Key words Streptococci M protein bactericidal test human IgG Fc anti human Ig Gm markers

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The laboratory methods recommended for the investigation of the virulence of group A streptococci include examination of mouse virulence and test of the ability of the strain to grow in normal human blood (13 14 17 25) The hyaluronic acid capsule – and in particular – the type specific antigens or M proteins are virulence factors of recognized importance (12 20) It is a widespread opinion that a strain which grows in normal human blood and is virul [illegible] contains M protein also when extracts of the strain fail to precipitate with type-specific antisera. Nevertheless only type specific antibodies are generally assumed to promote phagocytosis in human blood It is a matter of discussion whether group A streptococcal strains might be entirely devoid of M protein or substances closely resembling the M protein (9 10) Hence the term »absence of M protein« refers to the functional absence of M protein as defined by the tests for virulence referred to above

In addition to the M protein and the streptococcal capsule some other, hitherto unknown structures might be important for streptococcal virulence (1) Recently, it was found (5) that many group A streptococci have receptors for the Fc fragment of IgG the solubilized receptors display a variation in affinity between streptococcal types, e g with respect to reactivity for the human IgG1 and IgG3 subclasses (3) It remains to be investigated whether these receptors which are distinct from the M protein (4) could interfere with antibody-mediated phagocytosis

One of the usual procedures for reestablishing the M protein production of a strain is to pass the bacteria serially through mice and simply follow the presumed acquisition of M antigen by testing these passaged strains for growth in normal human blood (13) The present investigation concerns the changes observed in a type 12 group A streptococcal strain during serial mouse passages In contrast to the concepts about M protein and virulence referred to above it was found that the type 12 antigen was lost during the passages although the virulence for mice and the ability to grow in human blood increased During the passages the bacterial content of receptors for the Fc part of human IgG also increased

MATERIALS AND METHODS

Bacterial Strains and Extracts

Group A streptococci type 12 (strain 1800 from the collection at the Institute of Hygiene and Epidemiology Prague) were used throughout the study Hot hydrochloric acid extracts were prepared from over night cultures of the streptococci on 300 ml Todd Hewitt broth as previously described (3)

Mouse Passages of the M12 Strain

This method has been described previously (2) In brief 0 5 ml of over night culture of streptococci was injected intraperitoneally in white mice of 10–12 g After each mouse had died from septicemia a suspension of the spleen was injected intraperitoneally in a new mouse etc After each mouse passage the strain was isolated and designated according to the number of mouse passages performed before isolation *e g* the strain isolated after 15 passages was called M12/15

The experiments described in this paper were confirmed in two repeated series of mouse passages with strain 1800

Test of Streptococcal Strains for Mouse Virulence

White adult mice were inoculated intraperitoneally with 0 5 ml of tenfold dilutions 1 10^{-1} to 1 10^{8} of an over night culture of the test strain Five mice were injected with each suspension and the animals were observed for 3 days after which the number of deaths was registered The number of colony forming units (CFU) killing all mice was calculated (LD_{100})

Rabbit Sera

Two rabbit antisera to type 12 group A strep strain 1800 were prepared one in Leningrad other in Lund (Lu) Furthermore antiserum to type M strain 40/58 was prepared in Leningrad These were used unabsorbed Rabbit M typing sera again type 1 and type 12 group A streptococci from th Institute of Hygiene and Epidemiology Prague (Pr) we also used as was anti type 12 M typing serum kind supplied by Dr *El Kholy* Cairo (Ca)

Antisera to three strains originally types 3 12 and but devoid of type specificity after hundreds of mou passages (2) were also raised in rabbits These sera we designated anti 3P anti 12P and anti 46P respectivel The immunization schedule for the production of the and other antisera used followed the description g ven l *Rotta et al* (21)

The T typing sera were purchased from Chemap Prague

Bactericidal Tests

These tests were performed essentially as described Lancefield (13) and *Šramek* (25) In brief a measu quantity of streptococci (the inoculum) was mixed w freshly drawn human blood alone or in the presence added serum and incubated for 3 hours in rotating tub The growth index (25) indicating the degree of inocul propagation in normal human blood was calcula from the formula

$$\frac{\text{no of CFU obtained with normal serum}}{\text{no of CFU in inoculum}}$$

The bactericidal index (BI) indicates the degree inoculum growth inhibition in the presence of a streptococcal serum as compared to controls with nor serum (24) This index was calculated from the form

$$\frac{\text{no of CFU obtained with normal serum}}{\text{no of CFU obtained with antiserum}}$$

The scale used for expressing BI values was BI below 0 51–100 + 101–200 ++ 201–500 +++ above 500 ++++ (24)

Assay

barbital buffer pH 8 6 containing 2 mM calcium la was mixed with 0 8 ml anti type 12 serum Lu poured over a 205 × 110 mm glass plate Circular w taking 5 µl extract were cut 80 mm from the proje anode After application of the extract a current of 6 cm was applicated for 16 h The plate was stained w Coomassie Blue

or Streptococcal IgG Fc Receptors

se tests were performed essentially as described usly (3) In brief Rh positive red blood cells were with incomplete anti Rh antibodies from s individuals The capacity of different dilutions of

ubclass. Anti Rh KM and 317 are useful for on of the human IgG1 genetic markers G1m(1) 1m(4), respectively The anti Rh HUN detects the marker G3m(5)

le Immunodiffusion in gel

d precipitation test were performed as earlier ribed (23)

ntitation of Protein

The protein content of the extracts was quantitated by odified Folin method (16)

RESULTS

anges in the Virulence of the Streptococcal Strain ring Mouse Passages

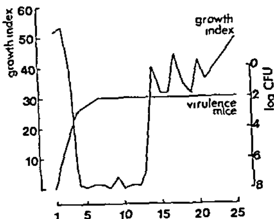
The growth index (which indicates the ability of a eptococcal strain to survive in human blood in tating tubes) dropped from 52 after the first ssage to one after four passages The strains 12/5 to M12/13 also showed very low growth dices (Fig 1) After 14 ...

tain inc ...

onstant ...

ssages (see Table 1)

After each passage the isolated strains were also sted for mouse virulence The results obtained owed increasing virulence during the first four ssages the LD_{100} dropped from 10^8 to 10^3 CFU During the following three passages the virulence was further increased After the 7th passage the irulence stabilized and remained constant showing LD_{100} between 10 and 100 CFU



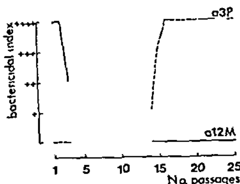


Fig 1 Changes in virulence for mice and behaviour in bactericidal tests of a type 12 group A streptococcal strain during serial passages through mice Abscissa no of passages performed Ordinate the virulence for mice given as the number of colony forming units (CFU) killing all of 5 mice ...

index which indicates the capacity of a serum to kill the streptococci in the presence of normal human blood was calculated according to *Stollerman et al* (24) a3P indicates the results with a rabbit antiserum to strain 3P and a12M the results with a rabbit antiserum to type M 12 (the Le serum)

TABLE 1 *Representative Bactericidal Tests with Some Selected Strains Obtained after Mouse Passages of Strain 1800*

	Bactericidal test with strains					
	M12/1	M12/2	M12/3	M12/14	M12/19	M12/25
No of CFU inoculated	105	93	93	55	86	106
Growth index	52	53	41	40	31	48
Bactericidal index						
-anti-type 12 serum	>5488	1249	137	1	0	1
-anti-3P serum	0	1	1	85	>2733	5168

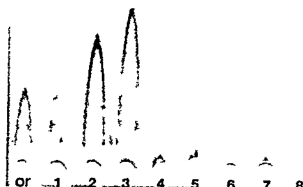


Fig 2 Electroimmunoassay with hot hydrochloric acid extracts of strain 1800 before and after passages through mice Twenty ml agarose was mixed with 0 8 ml of the anti type 12 serum Lu and poured over a glass plate Extracts were placed in the circular wells and after application of the current for 16 hours and washing the plate was stained with Coomassie Blue »or‹ indicates the precipitate obtained with strain 1800 and 1 2 3 etc the results of testing strains M12/1 M12/2 M12/3 etc The extracts of strains M12/10–M12/25 not shown on the picture did not give any precipitates

Loss of Type Specificity During the Mouse Passages

The Ca Le and Lu anti type 12 sera were compared by immunodiffusion in experiments with the hydrochloric acid extracts of the strains isolated after each of the 25 passages All three sera gave a strong precipitate against each of the extracts of M12/1 M12/2 M12/3 as well as the original strain When the precipitates were compared with one another Ouchterlony type I reactions (interference with complete fusion) were obtained between all lines This precipitate was not obtained with any of the extracts from the strains M12/4 to M12/25

Figure 2 shows the results of electroimmunoa say quantitation of the M protein content in t hydrochloric acid extracts of the passaged strain The height of the › rockets increased during th first three passages However the M prote precipitate disappeared and did not recur after t fourth passage

The protein content in the hydrochloric ac extracts of the passaged strains varied from 18 to mg/ml There was no correlation between u protein content of the extracts and the passages performed

T typing (11) of the passaged strains M12/1 - 7 9 10 and 25 was performed and all these strai were found to belong to T type 12 The other strai were spontaneously agglutinating and T typi could therefore not be performed

The loss of M12 protein was also confirmed the indirect bactericidal test using the anti type sera Le and Pr The bactericidal index (wh indicates the ability of an immune serum to kill streptococcal strain in the presence of fresh blo (24)) was estimated as ++++ after two and ++ after three Because of the low grow index it was not possible to determine t bactericidal effect of the anti type 12 sera on strai M12/4 to M12/13 The strains M12/14 to M12/ gave bactericidal indices with anti type 12 sera estimated as one (Fig 1)

Recently a suitable donor for bactericidal te with strain M12/6 (growth index 53) was fou (*Havlíček* personal communication) The strain w inhibited neiher by anti M12 nor by anti M serum

Test of the Passaged Strains for Streptococcal I Fc Receptors

The extracts of the passaged strains were test for capacity to agglutinate Ri sensitized human r

TABLE 2 *Agglutinat on of O Rh + Red cells Coated with Human IgG Anti Rh by Lancefeld Extracts of Type Group A Streptococci Subjected to Mouse Passage*

Human IgG coat	Genetic marker detected by the coat	Titre of the extract of type 12 group A streptococci after mouse passage no						
		0	6	7	11	13	23	25
Ri (1 15)*		1 10	1 80	1 20	1 160	1 160	1 320	1 320
KM (1 5)*	G1m(1)	1 4	0	0	1 4	1 4	1 4	1 4
317 (1 5)*	G1m(4)	1 2	0	0	0	0	0	0
HUN (1 10)*	G3m(5)	1 10	1 10	1 10	1 20	1 20	1 40	1 80

* Indicates the dilution of the anti Rh at coating

TABLE 3 *Summary of the Results of Bactericidal Tests and Virulence Tests*

uns	Virulence for mice	Growth index[a]	type 12M protein	Bactericidal index using anti M12 serum	anti-3P 12P and 46P sera
2/1 -3	low/intermediate	high	present	high	one
2/4 -13	high	zero	absent	n.d [b]	n d [b]
2/14-25	high	high	absent	one	high

he same blood donor used throughout the study

ot done because of low growth index

ls The titres did not exceed 1 10 in the extracts of e original strain, M12/1, M12/2 and M12/3 e titres of the extracts of M12/5 –9 varied tween 1 20 and 1 80 From M12/11 to M12/25 e titres increased and stabilized between 1 160 and 320 (Table 2)

The hemagglutinating capacity of nine of the strains was tested in experiments with red cells ated with KM, 317 or HUN »incomplete« anti- . The HUN coat useful for detection of the

acteridal Effect of Antisera against Strains peatedly Passaged in Mice and of Antiserum to

... originally belonging to types M3, M12 and M46 but devoid of type antigens after mouse passages The effect of anti 3P in indirect bactericidal test is shown in Fig 1 and in Table 1 similar results were obtained with anti-12P, anti-46P and anti M1 sera. These sera had no effect in the test with M12/1–3 However, a high bactericidal index was found for the strains M12/14–25

DISCUSSION

During the mouse passages, several characteristics changed in the type 12 group A streptococcal strain which confirm and extend previous findings (2) Table 3 summarizes the results of some of the experiments It appears that three major phases represented by strains M12/1–3, M12/4–13 and M12/14–25, ... passages Clear-cut differences were observed between strains M12/1–3 as compared to strains M12/14–25, including disappearance of M12 antigen, concomitant increase in mouse virulence and susceptibility in bactericidal test using antisera against heterologous types

The disappearance of M12 antigen after the fourth passage was observed in the precipitation studies and also in the indirect bactericidal test using anti-type M12 sera The mouse virulence increased after four passages and remained high during the following passages In contrast to the increasing mouse v...

although each strain was tested with blood from the same human donor Discrepancies between virulence for mice and behaviour in bactericidal tests as found for strains M12/4–13 have also been noted in some group A streptococci by *Todd* (26) and *Lancefield* (13)

Conceivably, at least three explanations may be given for the changes in the characteristics of strain 1800 during mouse passages

1) The strain 1800 might be a mixture of two M-types, e g M12 and M1, since anti M1 serum was bactericidal for strains M12/14–25 Hence, the M1 strain might have been selected because of its higher mouse virulence This would also be consistent with the finding that M1 has high IgG Fc receptor activity (6) However, this hypothesis implies also that the M3 and M46 strains were contaminated with type M1 since antisera raised against these strains after mouse passages were bactericidal for strains M12/12–25 This objection is valid also for suspected »contamination« of the type 12 strain with any other M type Studies involving clones from single cells of strain 1800 are in progress in order to further exclude the possibility of contamination

2) The changes in strain 1800 could be caused by

a shift in M type *Maxted & Valkenburg* (18) described an epidemic in which type 12 group A streptococci showed »antigenic drift« towards type 22 However »antigenic drift« occurring during the mouse passages would imply that the M antigens of three types M3 M12 and M46 had changed to one and the same type antigen during the passages i e type 1, since anti M1, 3P, 12P and 46P were all bactericidal for strains M12/14–25

3) Mouse passages of some group A streptococci of defined M type might occasionally lead to selection of variants which lack M antigen but have a hitherto unknown virulence factor in common *Wittner* (27) using strains which had been serially passed through mice found that purified M proteins had the capacity to absorb opsonic antibody from a variety of heterologous antisera prepared against whole cells or purified M proteins This absorption also separated passive mouse protecting antibodies and passive hemagglutinating antibodies from opsonic antibodies The author interpreted these observations as caused by shared determinants among M proteins of different serotypes However *Fischetti* (7) (who did not use mouse passaged strains) found only limited structural relatedness among three purified M proteins examined and found no evidence of an anti phagocytic activity caused by a peptide sequence common to M proteins *Fleck* (8) found anti type 6 and anti type 1 serum to protect mice against infection with type 18 streptococci suggesting »an antibody other than M which could protect mice and enhance phagocytosis«

Several reports have indicated the existence of other virulence factors than the hyaluronic acid capsule and M protein *Becker et al* (1) found that mouse passaged strains were rich in M protein and hyaluronic acid and concomitantly virulent for mice However group A streptococci which had been selected after incubation with human blood

[illegible]

discrepancy with respect to mouse virulence *Becker et al* (1) suggested the existence of as yet unidentified streptococcal virulence factors in addition to M protein and capsule We have found that the M12/25 strain retained the virulence for mice

[illegible]

tion)

Any pathogenic significance of the streptococcal receptors for human IgG Fc fragment has not so far been shown However increase in reactivity for IgG Fc fragment was concomitant with increase in mouse virulence in strains passed on mice In this context it is of interest that *Perkins & Hahn* (1[illegible]) found the phagocytosis of group A streptococci [illegible] human blood to be enhanced by antibody [illegible] derived from rabbit immunoglobulins by pepsi[n] digestion as compared with undigested antibod[y] *Saito* (22) digested rabbit streptococcal anti [M] antibody with papain the digested univalent anti body retained the indirect bactericidal activity [in] *vitro* and was as effective as intact anti M antibod[y] Thus the Fc fragment of IgG does not contribute [to] the phagocytosis of group A streptococci in contra[st] to the phagocytosis of other bacteria (19)

In conclusion loss of original M antige[n] concomitant with increasing virulence for mou[se] has been demonstrated in a type 12 group [A] streptococcal strain during mouse passages An[ti] sera against type M1 and three other hetero[logous] types subjected to mouse passages were fou[nd] bactericidal for the strains obtained from passag[es] of the original type 12 strain The nature of t[he] structure responsible for virulence of the passag[ed] strain is not known However the possible role [of] the streptococcal IgG Fc receptor should be cons[ide]red

We would like to thank Dr Jiři Havlíček Prague, [for] performing some tests and for helpful comments [and] suggestions

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a shift in M type *Maxted & Valkenburg* (18) described an epidemic in which type 12 group A streptococci showed »antigenic drift« towards type 22 However, »antigenic drift« occurring during the mouse passages would imply that the M antigens of three types, M3, M12 and M46 had changed to one and the same type antigen during the passages i e type 1, since anti-M1, 3P, 12P and 46P were all bactericidal for strains M12/14–25

3) Mouse passages of some group A streptococci of defined M type might occasionally lead to selection of variants which lack M-antigen but have a hitherto unknown virulence factor in common *Wittner* (27) using strains which had been serially passed through mice found that purified M proteins had the capacity to absorb opsonic antibody from a variety of heterologous antisera prepared against whole cells or purified M proteins This absorption also separated passive mouse protecting antibodies and passive hemagglutinating antibodies from opsonic antibodies The author interpreted these observations as caused by shared determinants among M proteins of different serotypes However, *Fischetti* (7) (who did not use mouse passaged strains) found only limited structural relatedness among three purified M proteins examined and found no evidence of an anti phagocytic activity caused by a peptide sequence common to M proteins *Fleck* (8) found anti type 6 and anti type I serum to protect mice against infection with type 18 streptococci suggesting »an antibody other than M, which could protect mice and enhance phagocytosis«

Several reports have indicated the existence of other virulence factors than the hyaluronic acid capsule and M protein *Becker et al* (1) found that mouse passaged strains were rich in M protein and hyaluronic acid and concomitantly virulent for mice However group A streptococci which had been selected after incubation with human blood were not virulent for mice although their content of M protein and hyaluronic acid was as high as found in mouse passaged strains On background of the discrepancy with respect to mouse virulence *Becker et al* (1) suggested the existence of as yet unidentified streptococcal virulence factors, in addition to M protein and capsule We have found that the M12/25 strain retained the virulence for mice also after and during injection in the test animals of hyaluronidase thereby removing the capsule of the streptococci (*Burova & Ryc*, unpublished observation)

Any pathogenic significance of the streptococcal receptors for human IgG Fc fragment has not so far been shown However, increase in reactivity for IgG Fc fragment was concomitant with increase in mouse virulence in strains passed on mice In this context, it is of interest that *Perkins & Hahn* [illegible] found the phagocytosis of group A streptococ[illegible] human blood to be enhanced by antibody [illegible] derived from rabbit immunoglobulins by [illegible] digestion as compared with undigested antib[illegible] *Saito* (22) digested rabbit streptococcal an[illegible] antibody with papain, the digested univalent [illegible] body retained the indirect bactericidal activit[illegible] *vitro* and was as effective as intact anti M antib[illegible] Thus the Fc-fragment of IgG does not contribu[illegible] the phagocytosis of group A streptococci [illegible] to the phagocytosis of other bacteria (19)

In conclusion, loss of original M [illegible] concomitant with increasing virulence for m[illegible] has been demonstrated in a type 12 grou[illegible] streptococcal strain during mouse passages [illegible] sera against type M1 and three other heterolo[illegible] types subjected to mouse passages were f[illegible] bactericidal for the strains obtained from pass[illegible] of the original type 12 strain The nature of [illegible] structure responsible for virulence of the pass[illegible] strain is not known However, the possible ro[illegible] the streptococcal IgG Fc receptor should be con[illegible]red

We would like to thank Dr Jiří Havlíček Prague [illegible] performing some tests and for helpful comments [illegible] suggestions

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SEROLOGY OF *CAMPYLOBACTER FETUS SS JEJUNI* (»RELATED« CAMPYLOBACTERS)

Demonstration of Strain-specific and Interstrain related Antigens by Immunoelectrophoresis and Co-agglutination

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osunen, T U Danielsson, D & Kjellander J Serology of *Campylobacter fetus* ss *jejuni* (»Related« ampylobacters) Demonstration of strain specific and interstrain related antigens by immunoelectrophoresis and co agglutination. Acta path microbiol scand Sect. B 88 207–218 1980

abbit antisera against two strains of *Campylobacter fetus* ss *fetus* (serotype A) two strains of *C fetus* s *intestinalis* (serotypes A and B respectively) and eight stains of *C fetus* ss *jejuni* were used in [illegible] it [illegible] d

onicates unheated boiled or autoclaved in R LIE The antigenic properties of *C fetus* ss *fetus* and *C fetus* ss *intestinalis* were distinctly different from those of the thermophilic *C fetus* ss *jejuni* strains as shown both by COA and L-RIE Serotypes A and B of the two former species were also differentiated In COA tests the *C fetus* ss *jejuni* organisms gave the strongest reactions with homologous antibodies but several interstrain cross reactions were seen By absorption strain specific COA reagents were obtained. Several reactions of identity indicating cross reactive antigens were also seen with L RIE within the ss *jejuni* group These results generally agreed with those obtained by COA With the use of unheated boiled or autoclaved organisms or sonicates heat labile antigens were differentiated from heat stable ones with the use of COA and R LIE The apparent antigenic heterogeneity of *Campylobacteria* indicates the importance of their serological grouping e g for clinical and epidemiological investigations COA and immunoelectrophoresis techniques can be effectively applied for such studies

Key words Campylobacter antigens Campylobacter serology co-agglutination immunoelectrophoresis

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Campylobacters originally classified as *Vibrios* have proven to be a heterogenous group of organisms. The species according to *Smibert* (11) are *C fetus* ss *fetus* (originally called *Vibrio fetus*) and *C fetus* ss *intestinalis* both of which are associated with infectious infertility and abortion in cattle and sheep and *C fetus* ss *jejuni* associated with diarrhoea in man and certain animals (4) The natural habitats of [illegible] former species are the genital and intestinal tracts of cattle and sheep (12) and of *C fetus* ss *jejuni* the intestinal tract of various animals including birds (4) The thermophilic properties of this latter species are an important marker to differentiate them from other pathogenic *Campylobacters*

The discovery that *C fetus* ss *jejuni* (»related« Campylobacters) is a common cause of enteritis in man (4 10) has greatly increased the interest in this genus and a need for improved classification has

become evident Serological classifications, which have greatly aided the understanding of the epidemiology, ecology and pathogenesis of infections with organisms as *Salmonellae, Shigellae Escherichia coli* and *Neisseria meningitidis* have been tried by some authors Based on autoclaving resistant antigens *Berg et al* (2) divided the *Campylobacters* into three main groups by tube agglutination Slide agglutination of live organisms and complement fixation tests of *C fetus* ss *fetus* strains have also been used (3) Strain specific and common antigens as well as non reactive strains were observed Thus, these studies, and those of the immune response of infected humans (4) have demonstrated complexity and heterogeneity

Co agglutination (COA) and immunoelectrophoresis techniques were recently applied for grouping and typing of *Neisseria meningitidis* (6) and *Neisseria gonorrhoeae* (8, 9) In the present study we have used a similar approach for a serological differentiation of *Campylobacter* strains the main emphasis being on strains from human enteritis patients

MATERIALS AND METHODS

Campylobacter Strains

Twelve strains were used in the study two *C fetus* ss *fetus* two *C fetus* ss *intestinalis* (»Montana« strains by the courtesy of Dr *Firehammer*) and eight of *C fetus* ss *jejuni* strains The designations sources and origins of the strains are listed in Table 1

The »Montana« strains grew well at + 37 and 25 °C but not at + 43 °C All the other strains grew at + 43 °C and 37 °C but not at + 25 °C All strains could be cultured on the selective medium described by *Skirrow* (10) in 5% O_2 atmosphere but not aerobically or anaerobically They were oxidase and catalase positive Gram negative bacteria which under the microscope fulfilled the morphological criteria laid down by *Sm*[...] (11)

Preparation of Antigens

The *Campylobacter* strains were grown on bo[...] blood agar plates in an atmosphere of 5% O_2 10% C[...] and 85% N_2 for 48 h The *C fetus* ss *fetus* and *C f*[...] ss *intestinalis* strains were incubated at + 35 °C, others at + 43 °C

Antigens for immunization The organisms [...] harvested in sterile saline containing 0 5% form[...] adjusted to a concentration of approximately 4–5 × [...] organisms ml^{-1} according to the Mc Farland scale E[...] parts of this suspension and complete Freund s adju[...] were mixed and used for immunizations

Antigens for absorptions and co agglutination ([...] *tests* The organisms were harvested in 0 15 M s[...] (PBS) centrifuged down at 3000 × *g* for 20 min, adjusted to a 10% (w/v) concentration After [...] treatment at + 56 °C for 30 min the whole bacteria [...] used for absorptions either as such (L) treated [...] formalin (F) as described (5) boiled in a water bath [...] h (B) or autoclaved at + 120 °C for 2 5 h (A[...] absorptions the suspensions were centrifuged at 30[...] *g* for 20 min and adjusted to a concentration of 25[...] ml^{-1} (wet weight) Formalin treated cells (F) were [...] for absorptions only

For COA tests the 10% suspensions (L, B a[...] were diluted further with PBS to a concentration o[...] (wet weight) (COA tests with live organisms be[...] like those treated at + 56 °C This treatment killed [...] than 99% of the organisms and was made [...] precaution Such organisms were therefore conside[...] contain both heat labile and heat stable antigens)

Antigens for immunoelectrophoresis The orga[...] were harvested in PBS centrifuged down at 3000 [...] for 20 min suspended in PBS at a concentration o[...] mg ml^{-1} (wet weight) and disrupted by sonication [...] watt MSE apparatus was used The disruption [...] accomplished by sonication at maximal efficiency [...] to eight periodic intervals of 20 s The sonicates [...] used either as such (for line rocket IE and rocket i[...]

TABLE 1 *Campylobacter Strains Used in the Present Study*

Species and identification No	Serotype	Source of isolation	Obtained from
C fetus ss *fetus* 13823	A 1	Bovine fetus	Bozeman Mon[...]
C fetus ss *fetus* 14840	A subtype 1	Bovine cervicovaginal mucus	Bozeman Mon[...]
C fetus ss *intestinalis* 13014	A 2	Ovine fetus	Bozeman Mon[...]
C fetus ss *intestinalis* 14865	B	Bovine fetus	Bozeman Mon[...]
C fetus ss *jejuni* VTP 67		Pig	Örebro Sweden
C fetus ss *jejuni* 372		Human feces	Helsinki
C fetus ss *jejuni* 113		Human feces	Helsinki
C fetus ss *jejuni* 105		Human feces	Helsinki
C fetus ss *jejuni* 441		Human feces	Helsinki
C fetus ss *jejuni* 341		Human feces	ATCC
C fetus ss *jejuni* ATCC 29428		Human feces	Malmo Sweden
C fetus ss *jejuni* ST 8			

r 1 h (for rocket line IE) or autoclaved for 2 5 h
ket line IE)

in ation White adult rabbits were immunized
th one of the 12 *Campylobacter* strains Initially
re injected subcutaneously with organisms mixed
mplete Freund s adjuvant. 1 5 ml and 1 0 ml in
nd and fore leg respectively A series of four
ous injections was started 4 weeks later (0 25
and 2 0 ml 3–4 days apart) The rabbits were
days after the last injection Both antisera and
une sera were stored in small aliquots at −40 °C
hawing merthiolate (1 10 000) was added to the
which was then stored at + 4 °C

p ons One volume of antiserum was mixed
ur volumes of whole *Campylobacter* organisms
ormalin treated boiled or autoclaved (see below
oelectrophoresis) at a concentration of 250 mg
wet weight) This mixture was incubated at + 37
3–4 h after which the serum was recovered by
ugation tw ce at 3000 × g for 20 min Absorbed
a were stored frozen at −40 °C and after thawing
or preparing COA reagents as described below

paration of reagents The production of protein A
lococci and the preparation of reagent staphylo
coated with unabsorbed or absorbed
ampylobacter antibod es as well as control rea
followed the procedure described before (6 7 9)

formance of COA tests One drop of a 1%
nsion (w/v) of reagent staphylococci was mixed on
s slide with one drop of the 1% *Campylobacter*

ons were recorded as and positive ones as 1 +
) 2 + (moderate) and 3 + (strong and very strong
ons)

ne rocket immunoelectrophores s (L RIE) This was
ed out as described by *Sandström & Dan elsson* (8)

s was about 15 cm² and the reference antigen
gel at the cathod c end of the plate c 10 cm² Each
angular agarose area with antigen of the intermediate
measured c 0 25 cm² They were each separated by a
spond ng rectangular agarose gel without antigen
rectangular gels we e made in a mould made up of
glass plates separated by a U shaped frame 45 ×
mm, 2 mm thick 0 3 ml agarose was carefully
red at the bottom (45 mm wide) of the mould After
dification 0 3 ml antigen-containing gel (0 05 ml
cated cells 100 mg ml⁻¹ and 0 25 ml agarose) was
red on the top and this procedure was repeated for
h antigen to be tested In this way sonicates of 20
mpylobacter strains or more could be layered on top of
h other The gel was removed from the mould and 8
n wide slices were cut perpend cular to the layers in
middle portion of the gel One such slice 2 × 8 ×
100 mm was lifted into place on the 100 × 100 mm
glass plate and placed 2 mm from the line antigen gel 10
× 100 mm This had been previously made by pouring a
mixture of 2 ml agarose and 0 3 ml sonicated
Campylobacter cells against a brass stopper 12 mm from
the cathodic end of the glass plate Contact gels c. 2 and
7 mm wide were poured after which the rest of the glass
plate 75 × 100 mm was covered with a mixture of 13
ml agarose and 0 5 ml *Campylobacter* antiserum

Rocket l ne immunoelectrophoresis (R LIE) This was
carried out essentially as described by *Axelsen et al* (1)
using the micro modification with 5 × 5 cm glass plates
Agarose and barbital buffer were the same as for L RIE
Antigen wells antigen containing intermediate gel and
reference anti-*Campylobacter* antibody gel were arranged
as shown in Fig 3 The areas covered by gel measured
approximately 7 5 5 and 12 5 cm² respectively
thickness 1 5 mm The antigen wells were filled with 10
µl of one of the sonicated antigens Various concentra
tions of the antigens were tested and 100 mg ml⁻¹ (refer
red to the wet weight of the organisms) gave optimal
results The intermediate gel contained 20 µl/cm² of
antigen preparations as indicated

Electrophoresis The barbital buffer in the electropho
resis vessels ionic strength 0 1 pH 8 6 was changed
after each run and the electrophoresis apparatus was
cooled with tap water during the test runs Electrophore
sis was run for 16 h with a gradient of 1 5 V cm⁻¹ The
plates were then pressed between filter papers washed in
saline and water pressed agains and stained with
Coomassie blue (1)

RESULTS

Co agglutination

Reagent staphylococci coated with unabsorbed
antibodies from individual immune sera against the
Campylobacter strains were tested against homolo-
gous and heterologous cells L B or A (See
Materials and Methods) The results of these tests
are given in Tables 2 & 3

The strongest COA reactions were always seen
with homologous organisms Three of the *C fetus*
ss *fetus* and ss *intestinalis* strains (serotype A
according to *Berg et al* (2) Table 1) were very
similarly reacting They differed markedly from *C
fetus ss intestinalis 14865* (serotype B according to
Berg et al (2)) These four strains (with lower
temperature optimum) were distinctly different
from the thermophilic *C fetus* ss *jejuni* strains and
between the groups only a few weak cross reactions
were observed

Among the thermophilic strains a close antigenic
relationship was noted between ATCC 29428 and
ST 8 Minor cross reactions were obtained with
some of the reagents and the heat stable and/or heat
labile antigens of other heterologous strains for
example between anti 441 and strains VTP 67 372
341 ATCC 29428 and ST 8

TABLE 2 *Co-agglutination Reactions of* C fetus ss fetus, C fetus ss intestinalis, *and* C fetus ss jejuni *Organisms in Tests with Reagent Staphylococci Coated with Unabsorbed Anti-*C fetus ss fetus *and Anti-*C fetus ss intestinalis *Antibodies*

Reagent staphylococci coated with anti-*Campylobacter* antibodies	Co-agglutination reactions obtained with *Campylobacter* organisms																	
	C fetus ss *fetus*						*C fetus* ss *intestinalis*						*C fetus* ss *jejuni*					
	13823			14840			13014			14865			105			VTP 67[b]		
	L[a]	B[a]	A[a]	L	B	A	L	B	A	L	B	A	L	B	A	L	B	A
C fetus ss *fetus* 13823	+++	+++	+++	+++	+++	++	++	+++	++	−	+	+	−	−	−	−	−	−
C fetus ss *fetus* 14840	+++	+++	+++	+++	+++	++	+++	+++	++	+	+	−	+	+	+	−	−	−
C fetus ss *intestinalis* 13014	+++	+++	+++	+++	+++	++	+++	+++	++	+	+	−	−	−	++	−	−	−
C fetus ss *intestinalis* 14865	−	−	−	−	−	−	−	−	−	+++	+++	+	−	−	−	−	−	−

[a] L = Organisms heated at 56 °C
B = Boiled organisms
A = Autoclaved organisms

[b] C *fetus* ss *jejuni* strains 372 113 441 341, ATCC 29428 and ST 8 gave identical results

Antisera against the thermophilic strains were ...d for absorptions with heated and non heated ...tigens The effectiveness of these absorptions was ...ted by homologous COA tests Absorptions with ...mologous non heated antigens completely remov... all reactivity Homologous absorptions with ...toclaved antigens removed reactivity against ...toclaved antigens and in some cases also against ...iled (Table 3 e g anti 441 absorbed with 441 A) ... this way the reactivity of heat labile antigens ...uld be differentiated from heat stable ones For ...me strains absorptions with autoclaved homolo...us organisms left reactivity against boiling ...sistant antigens (e g anti 105 absorbed with 105 ..., Table 3) Results after absorption with boiled ...tigens indicated that heat labile antigens were not ...sponsible

Unabsorbed antibodies against strains VTP 67 72 113 and 341 had very few cross reactions and ...ith simple heterologous absorptions strain specific ...tibodies were obtained Similar heterologous ...sorptions of antisera against strains 105 441 and ATCC 29428 which showed broader cross ...eactivity made it possible to make specific reagents also against these strains

Immunoelectrophoresis

LRIE as described by *Sandstrom & Danielsson* (8) was used to study the antigenic relationship between the various *Campylobacter* strains The arrangement of these tests is shown in Figs 1 & 2 The homologous antigen antibody system consisting of the antibody gel at the anode and the line antigen gel at the cathode with a small rectangular block of antigen-containing agarose perpendicular to the line antigen gel will under these circumstances form horizontal precipitin lines fusing with the rocket precipitin lines formed where the rectangular antigen-containing agar block is situated Comparison with antigens from heterologous strains can be

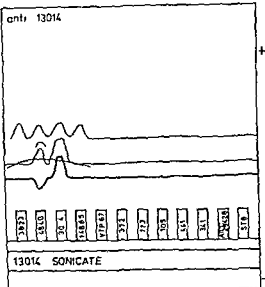


Fig 1 Schematic drawing of a line rocket immunoelectrophoresis with the antigen antibody combination of *C fetus* ss *intestinalis* 13014 with heterologous *Campylobacter* antigens in the intermediate gel The homologous antigens have formed horizontal precipitin lines fusing with the rockets formed by the homologous intermediate antigen and with rockets of the *C fetus* ss *fetus* and *C fetus* ss *intestinalis* group The thermophilic *C fetus* ss *jejuni* strains had no antigens reactive with this antiserum

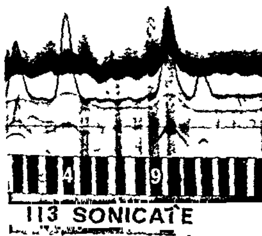


Fig 2 Line rocket immunoelectrophoresis with the antigen antibody combination of *C fetus* ss *jejuni* 113 with heterologous *Campylobacter* antigens in the intermediate gel The line antigens of the homologous strain have formed three complete horizontal lines fusing with rockets of the homologous antigens in the intermediate gel Additional rockets as well as an incomplete horizontal precipitin line (nearest the intermediate gel) fusing with small rockets were also formed by some of the heterologous *C fetus* ss *jejuni* and one of the *C fetus* ss *fetus* strains The antigens in the intermediate gel were (from the left) 1 105 2 CJ 10 (not reported further) 3 ATCC 29428 4 VTP 6 ... 8 6 ST 1 (not reported further) 7 372 8 441 ... 10 113 (homologous) 11 13014 12 1... and 14 14865

TABLE 2 *Co-agglutination Reactions of* C fetus ss fetus, C fetus ss intestinalis, *and* C fetus ss jejuni *Organisms in Tests with Reagent Staphylococci Coated with Unabsorbed Anti-*C fetus ss fetus *and Anti-*C fetus ss intestinalis *Antibodies*

Reagent staphylococci coated with anti-*Campylobacter* antibodies	Co-agglutination reactions obtained with *Campylobacter* organisms																	
	C fetus ss *fetus*						*C fetus* ss *intestinalis*						*C fetus* ss *jejuni*					
	13823			14840			13014			14865			105			VTP 67[b]		
	L[a]	B[a]	A[a]	L	B	A	L	B	A	L	B	A	L	B	A	L	B	A
C fetus ss *fetus* 13823	+++	+++	+++	+++	+++	++	++	+++	++	−	+	+	−	−	−	−	−	−
C fetus ss *fetus* 14840	+++	+++	+++	+++	+++	++	+++	+++	++	+	+	−	+	+	+	−	−	−
C fetus ss *intestinalis* 13014	+++	+++	+++	+++	+++	++	+++	+++	++	+	+	−	−	−	++	−	−	−
C fetus ss *intestinalis* 14865	−	−	−	−	−	−	−	−	−	+++	+++	+	−	−	−	−	−	−

[a] L = Organisms heated at 56 °C
B = Boiled organisms
A = Autoclaved organisms

[b] *C fetus* ss *jejuni* strains 372 113 441, 341, ATCC 29428 and ST 8 gave identical results

…isms of Campylobacter strains

105		441			341			ATCC 29428			ST 8		
B	A	L	B	A	L	B	A	L	B	A	L	B	A
		+			+						+		
					+								
+++	+++	+	+	++	+	+		+	+	++	+		
++					+								
+++	+++	+			+			+			+		
++	++												
		++	++	++			++			++	+		+
		+											
		+											
		+	+							+			
					+++	++	++						
					+	++							
	+	+++			+++			+++	+++	+++	++	+	+++
		++						+			+		
								+					
		++						+++	++	+++	++	+	+++
		+						+	++		+		
								++	+++	+++	+	+	++
+		++	++		+	+		+	++	++	++	+++	+++

…uns are given in Figs 1 & 2 and the results of all …uns are combined in Table 1

The antigen-antibody combinations for the *C fetus* ss *fetus* and *C fetus* ss *intestinalis* strains showed several cross reactions Strains 13014 and 14865 had specific antigens not shared by the other two ones Antisera against these four strains formed no precipitin lines with antigens from the thermophilic *C fetus* ss *jejuni* strains

Antisera against the thermophilic strains VTP 67 and 341 gave only one strong precipitin line fusing into the homologous rocket line In these runs a few cross reactions (CR) were seen with other strains studied but no rocket lines showing reaction of

TABLE 3 *Co-agglutination Reactions of Thermophilic* Campylobacter *Organisms Treated with Heat at 56° C (L), Boiled (B) or Autoclaved (A) Reagent Staphylococci were Sensitized with Unabsorbed or Absorbed Antibodies a Indicated in the Table Reactions Graded as Described in Materials and Methods*

Anti *Campylobacter* antibodies used for coating reagent staphylococci	Absorbing strain	Co agglutination reactions obtained[a]								
		VTP 67			372			113		
		L	B	A	L	B	A	L	B	A
VTP 67	None	+++	+++	+++		+	+			
	VTP 67-B	b)								
	VTP 67-A		+							
	372-A	+++	+++	+++						
372	None	++			++	++	+++	+		
	372 B									
	ST 8 L				+	++	++			
113	None	+						+++	+++	+
	113-B									
	105-L							+++	+++	+
105	None	+		+	+		+	+		+
	105-B									
	105-A	+								
	441-A	+			+			+		
	VTP 67-F[a)]									
441	None			+	+		++			
	441 B									
	441-A									
	341-A									
341	None									
	341-B									
	341 A									
ATCC 29428	None	++			++			++		
	ATCC B				+					
	ATCC B, 441-L									
	VTP 67 L				+					
	VTP 67-L, ST 8-F									
	VTP 67 L, 441-F									
ST 8	None				++		++	+		
	ST 8 B									

a) F = formalin treated organisms
b) Empty space means negative reaction

done by including antigens from these strains in rectangular agar blocks parallelly with the homologous antigen-containing agar block Cross-reacting antigens will form a rocket fusing with the horizontal precipitin line and rocket line of the homologous system These cross-reacting antigen-antibody lines are designated CX1, CX2 etc depending on the number of such lines Anoth type of cross-reactions can appear as a rocket lin not connected with a horizontal precipitin lin probably due to too low a concentration of th antigen in the line gel Such reactions and cros reactions are designated CR

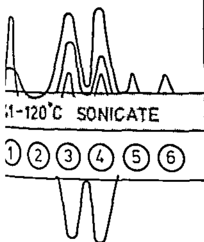
A schematic drawing . otograph of typic

C fe us ss jejuni ...	CR	CR	CR	CR	- -	CX2	CX1 CX2 CR	CX2	CX2 CR	CR	CX2 CR	CX2 CR	CX2 CR
C fetus ss jejuni 113	CX2 CR					CX2 CX3 CR	CR	CX1 CX2 CX3 CR	CX2 CX3 CR	CR	CX2 CR	CR	CR
fetus ss jejuni 105	CR	CR	CR	CR		CX2 CX3 CR	CX3 CR	CX2 CX3	CX1 CX2 CX3	CX3 CR	CX2 CX3	CR	CX3 CR
C fetus ss jejuni 441	CR	CR	CR	CR		CX3	CX2 CX3 CR	CX3	CX3	CX1 CX2 CX3 CR	CX3 CR	CX2 CX3 CR	CX2 CX3 CR
C fetus ss jejuni 341						CR		CR	CR		CX1		
C fetus ss jejuni ATCC 29428	CR	CR	CR	CR		CR	CX1	CX1	CX1 CR	CX1	CX1	CX1 CX2	CX1 CX2
C fetus ss jejuni ST 8	CR	CR	CR	CR			CR	CX1		CR		CX1 CR	CX1 CR

a) See text for abbreviations
b) Empty space means negative reaction

TABLE 4 *Results with the Line-rocket Immunoelectrophoresis with the Use of Anti-*Campylobacter *Antisera Tested against Sonicated Cells of Homologous and Heterologous* Campylobacter *Strains in the Intermediate Gel*

Antigen in line gel and anti-*Campylobacter* antiserum	L-RIE reactions with homologous and heterologous *Campylobacter* antigens in intermediate gel											
	C fetus ss *fetus*		*C fetus* ss *intest*		*C fetus* ss *jejuni*							
	13823	14840	13014	14865	VTP 67	372	113	105	441	341	ATCC 29428	ST 8
C fetus ss *fetus* 13823	CX1[a)] CX2 CX4	CX1 CX2 CX4	[b)] CX2 CX4	CX2 CX4								
C fetus ss *fetus* 14840	CX2 CX3 CX4 CR	CX1 CX2 CX3 CX4	CX1 CX3 CX4	CX2 CX3 CX4								
C fetus ss *intest* 13014	CX3 CX4	CX2 CX3 CX4	CX1 CX2 CX3 CX4	CX3 CX4								
C fetus ss *intest* 14865	CX2 CX4	CX2 CX4	CX2 CX4	CX1 CX2 CX4								
C fetus ss *jejuni* VTP 67												



t stable antigens gave precipitin lines By luding heated or non heated heterologous antigen the intermediate agar gel we could also show for ample that heat labile and heat stable antigens of TP 67 were cross reactive with 105 (Fig 3b) ditional heat labile and heat stable antigens were sent in 105 but not in VTP 67 These findings rrespond with the results of the COA reactions orrespondingly R LIE with the anti 441-441 mbination with 113 in the intermediate agar gel owed that 441 had at least two precipitating heat bile antigens in common with 372 ATCC 29428 d ST8 and one heat stable antigen not in mmon with these strains With similar R LIE ts we could also prove that 341 contained heat bile antigens reactive with anti ATCC 29428 which is in agreement with COA findings and that ST8 and ATCC 29428 shared heat stable antigens (Fig 3c) which was also found by COA test In these R LIE tests we also found that ATCC 29428 and ST8 shared an antigen which moved in the cathodic direction (Fig 3c) These findings correspond with the general results of the COA reactions There were however a few minor discrepancies

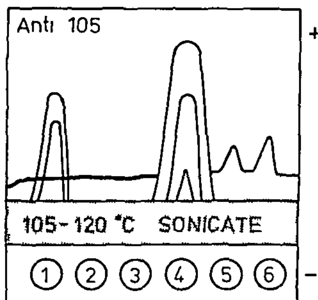
DISCUSSION

The thermophilic properties of *C fetus* ss *jejuni* are an important marker to differentiate them from other *Campylobacteria* However very few markers are available to divide the *jejuni* group further for e g clinical epidemiological studies *Berg et al* (2) found by tube agglutination tests that this group had an autoclaving resistant common antigen (designated »C«) and one out of seven heat labile antigens found in other *Campylobacteria* *Butzler* (3) also used agglutination for classification but due to the tendency of these organisms to spontaneously agglutinate or aggregate direct agglutination methods sometimes failed to give dependable results

Our data show that co-agglutination which is generally not influenced by spontaneous agglutination or aggregation (7) can be applied for serological studies of *Campylobacteria* By immunizing rabbits with formalin fixed bacteria we obtained antibodies which sensitized Cowan I staphylococci to co agglutinate live or heat inactivated (56 °C) boiled and autoclaved organisms Prepared reagents distinctly differentiated *C fetus* ss *jejuni* the thermophilic group from *C fetus* ss *fetus* and *C fetus* ss *intestinalis* The COA tests also differentiated subtype A from the subtype B strains of the *fetus intestinalis* group which is in agreement with the agglutination results reported by *Berg et al* (2)

Several cross reactions were revealed within the thermophilic group However heterologous absorptions of the antibodies demonstrated individual differences which indicates heterogeneity within this group The only pig isolate (VTP 67) was entirely different from the other strains while two strains ATCC 29428 and ST 8 were closely related The COA method would thus be suitable for assessing identity or non identity i e the epidemiological significance of isolates from outbreaks and differences in the distribution of serotypes from different geographical areas

Line rocket immunoelectrophoresis tests gave further support to the antigenic difference between *C fetus* ss *fetus* – *C fetus* ss *intestinalis* group and *C fetus* ss *jejuni* This technique as described by *Sandstrom & Danielsson* (9) allows comparison of



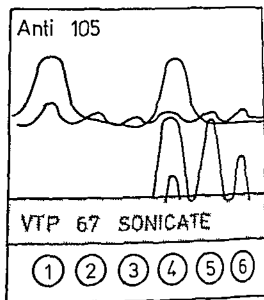


Fig 3a–c Schematic drawings of rocket line immuno electrophoresis tests with antigen-containing intermediate agar gel In 3a, the wells 1, 2 & 3 contain sonicated antigens heated at 56 °C boiled and autoclaved, respectively, of strain 441, the wells 4, 5 & 6 correspondingly treated antigens of strain 105 Heat labile antigens of strain 441 have formed 2 rockets The horizontal line formed by the antigen of strain 105 in the intermediate gel has fused with the rockets formed by boiled and autoclaved antigens of 105 in wells 5 and 6

In *3b* the wells 1 2 & 3 contain sonicated antigens heated at 56 °C boiled and autoclaved, respectively of VTP 67, the wells 4 5 & 6 correspondingly treated antigens of strain 105 The antigens of VTP 67 heated at 56 °C in the intermediate agar gel have formed 2 horizontal lines both of which fuse with rocket lines of heat labile antigens of VTP 67 and 105 Additional rockets were formed by heat labile and heat stable antigens of 105

In *3c* the wells 1 & 2 contain sonicated antigens heated at 56 °C and autoclaved respectively of strain 341 the wells 3 4 5 & 6 correspondingly treated antigens of strains ST 8 and ATCC 29428 respectively Strain 341 shared two heat labile antigens with the two latter ones Note the antigenic similarity of ST 8 and ATCC 29428 both of which also formed a rocket line with a reaction of identity with positively charged heat labile antigens

identity with the horizontal one The remaining antisera gave two to three rocket lines fusing with the horizontal lines formed by the homologous antigen Several reactions of identity were obtained with the other thermophilic strains, including VTP 67 and 341 This would indicate that also VTP 67 and 341 have several antigens cross-reacting with the other strains, but that their antisera were n[ot] optimally reactive to detect these cross reactions t[he] other way The close relation between the strai[ns] ATCC 29428 and ST 8, demonstrated in the CO[A] tests, is also obvious in the IE tests It should [be] noted that the homologous antigen antibody comb[i]nations of the other thermophilic strains alwa[ys] gave one more precipitin line than the cross reacti[ng] heterologous strains, thus representing strain spe[ci]fic reactions

Comparison of Results Obtained by Co-Agglutinat[i]on and Immunoelectrophoresis

By comparing Tables 2, 3 and 4 it can be se[en] that the COA results generally agreed with those [of] the L-RIE tests A few cross reactions wer[e] however obtained by the COA tests but not by t[he] L-RIE and *vice versa*

Both heat labile and heat stable antigens we[re] responsible for the COA reactions with the homolo[-] gous antigen antibody combinations It was als[o] found that cross reactive antigens demonstrated b[y] COA tests could be heat labile or heat stable (Table 2 and 3) Rocket line immunoelectrophoresis (R[-] LIE) with antigen-containing intermediate agar g[el] as described by *Axelsen et al* (1) was therefore use[d] to study the sensitivity to heat of homologous an[d] cross-reactive antigens demonstrated by L-RIE (Ta[-] ble 4) The arrangement of and results with such R[-] LIE tests are illustrated in Fig 3a–c

By including unheated or heated (100 or 120 °C homologous antigens in the intermediate agar ge[l] we could show (Fig 3a) that both heat labile an[d]

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ANTIVIRAL AND CELL MULTIPLICATION INHIBITORY ACTIVITIES OF MOUSE INTERFERON PREPARATIONS TESTED ON AN INTERFERON SENSITIVE MURINE SARCOMA CELL LINE

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gre, M Antiviral and cell multiplication inhibitory activities of mouse interferon preparations tested an interferon sensitive murine sarcoma cell line Acta path microbiol scand Sect. B 88 219-223 10

e cell multiplication inhibitory effect of SDS treated mouse interferon separated into antiviral (AV) f cell multiplication inhibitory (CMI) fractions was compared to that of untreated similar interferon a line of murine osteosarcoma cells The untreated interferon preparation and the CMI fractions se-dependently inhibited the multiplication of the cells as measured by cell count and incorporation of thymidine into the cultures The AV fractions containing comparable antiviral activities as the treated interferon preparations had only a minor effect on cell multiplication The biochemical operties of the fractions studied remain unknown

y words Interferon murine sarcoma osteosarcoma tissue culture

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ides its well-documented antiviral activity eron exerts several biological effects at the ar level (17) Among these »non antiviral« tes the antitumor effect (13) and the cell plication inhibitory effect (7 12 16) have been extensively studied Interferon preparations it the multiplication of both normal and tumor *in vitro* (2 7 12 15 16) and the development imors *in vivo* (13) in several experimental els. Glasgow and co-workers (3 11) reported interferon inhibited murine osteosarcomas *in* and *in vivo*

n most studies the antiviral and the cell tiplication inhibitory activities of interferon elated strictly However separation of these vities has also been *reported first* by affinity omatography on an albumincoupled agarose column (6) and subsequently by boiling interferon preparations in the presence of sodium-dodecyl sulfate (SDS) followed by gel filtration on a Sephadex G 25 column (4)

In the present study the effects of fractions obtained by the SDS method on murine osteosar coma cells *in vitro* have been studied

MATERIALS AND METHODS

Cells

The continuous line of mouse L-929 fibroblast cell originally obtained from the American Type Culture Collection, Rockville Md was grown in Eagle's minimal essential medium (MEM) supplemented with 5-10 per cent fetal bovine serum 300 µg glutamine 100 µg penicillin and 50 µg streptomycin per ml

antigens of several strains (20 or more) in one and the same test run Several cross reactive antigens were demonstrated within the *jejuni* group However, the individual antigenic characteristics demonstrated by COA tests were also found by L-RIE and further documented by R-LIE tests with the antigen-containing intermediate ager gel technique

COA and IE tests with unheated and heated antigens further documented the antigenic complexity of the *Campylobacteria* Homologous absorption of the antibodies with autoclaved antigens allowed a differentiation of heat stable antigens from heat labile ones by COA tests These findings were confirmed by R-LIE tests which also showed that several heat sensitive and heat resistant antigens participated in the reactions It was also of interest to note that absorptions with boiled bacteria removed reactivity in most cases also against organisms inactivated at 56 °C (Table 2) Whether this is an indication of the presence of capsular (K) antigens in *Campylobacteria* (13) remains to be shown

The close antigenic relationship between ATCC 29428 and ST 8, demonstrated by COA, was confirmed by immunoelectrophoresis which showed that these strains were the only ones with antigens that moved in the cathodic direction These findings as well as those with the COA method prove the suitability of these techniques for further serological studies and antigenic analysis of *Campylobacteria*

This study was aided by grants from the *Sigrid Juselius Foundation* Helsinki and the *Research Foundation of the Örebro County* Örebro Sweden The skillful technical assistance of Mrs *Eila Kelo Maire Laakso Angela Andersson* and *Tuula Nykanen* is gratefully acknowledged

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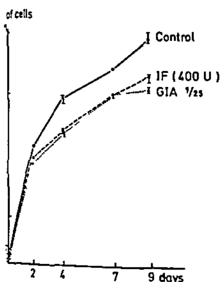
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.2 Growth of osteosarcoma cells in the presence of ıferon and the CMI fraction Vertical bars indicate 1

dissolved in 5 ml Aquasol The radioactivity in the filterpads was determined with a Mark II liquid scintillation spectrometer (Nuclear Chicago Corp Des Plaines Il) Cell counts were determined in parallel cultures after trypsin treatment in a haemocytometer

RESULTS

The SDS treated interferon preparation was fractionated on a Sephadex G-25 column, and eluted with distilled water in 3 ml *fractions* Each fraction was tested on L cells for antiviral activity and effect on cell multiplication (Fig 1) Fractions 6 and 7 contained the major part of AV activity and fractions 13 and 14 the CMI activity The interferon titre of the AV pool (fractions 6 and 7) was 10 000 units per ml and of the CMI pool (fractions 13 and 14) 25 units per ml

Osteosarcoma cells were cultured in the presence of pooled AV or CMI fractions or interferon preparation After 3 days' incubation the cell counts were determined The data presented in Table 1

ig 3 Uptake of ^3H thymidine into osteosarcoma cells after incubation with CMI and AV fractions for 24 and 72 ours Vertical bars indicate 1 SD

A mouse osteosarcoma cell line established from a ^{239}Pu irradiation produced primary tumor in C57B1/6 mice (11) The osteosarcoma cells were grown in the same medium as the L cells In the present experiments the cells were in their 50–70th passage in cell culture The cells remained tumorigenic when injected into C57B1/6 mice

Interferon

Murine type I interferon preparation, produced by Dr *K Paucker*, Medical College of Pennsylvania, Philadelphia, Pa, by exposing L- cells to Newcastle disease virus (NDV), was received from the antiviral program of the National Institute *of Allergy and Infectious Diseases*, Bethesda, Md The specific activity of this preparation was about 1×10^7 units per mg protein Another preparation, produced in L- cells by NDV inoculation, was given to us by Dr *G Bodo*, E Boehringer Institut fur Arzneimittelforschung, Vienna This preparation contained $1\ 3 \times 10^6$ units of interferon per mg protein Antiviral activity was measured against Vesicular stomatitis virus (VSV) in L cells by a plaque reduction method (14) or by an infectivity inhibition microtest (5) All titres were compared to international reference standards

Interferon fractions containing antiviral (AV) and cell multiplication inhibitory (CMI) activities, prepared by SDS treatment, were obtained from *H Dahl*, Wilhelmsen Institute, University of Oslo Briefly, interferon preparation was treated with $3\ 3 \times 10^{-3}$ M SDS in boiling water bath for 1 minute After 1 hour at room temperature the preparation was gel filtrated on Sephadex G-25 *column, and the antiviral and cell multiplication* inhibitory activites were tested in each fraction (4, 7) The active fractions were pooled for further studies

Determination of Cell Proliferation

Osteosarcoma cells were seeded in 24 well plates (Linbro), 5×10^3 cells in each well Interferon or interferon fractions obtained by SDS treatment were added simultaneously and incubated at 37 °C After various times of incubation the cells were trypsinized, and the number of viable cells was determined after trypan blue staining in a haemocytometer Each value given in the results is the mean of three or four determinations Other cultures were fixed with 20 per cent formalin at the end of incubation, washed and stained with methylene blue

Incorporation of ^3H-thymidine (Tdr) into Cell-cultures

Osteosarcoma cells were seeded in 96 well plates (Linbro), about 5×10^3 cells into each well Interferon or interferon fractions were added simultaneously The cells were incubated at 37 °C for 24 or 72 hours At each time period 1 µCi ^3H-Tdr (40–60 µCi/mM, New England Nuclear, Boston, Ma) was added to each well and incubated for 4 hours The supernatant was then discarded, the cells were washed with phosphate buffered saline (PBS), trypsinized and harvested onto filter pads with the aid of an automated cell harvester (Scatronic, Flow Laboratories Inc , Inglewood, Ca) Filterpads were

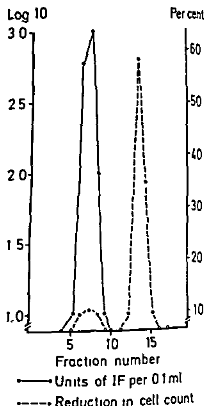


Fig 1 Antiviral (AV) and cell multiplication inhib (CMI) activities of fractions obtained from gel filtr on Sephadex G 25 of mouse interferon treated sodium dodecyl sulfate at 100 °C for 1 minute L cells were grown in the presence of fractions for 3 Each fraction was tested at dilution 1 25
●———● *Units of interferon* per 0 1 ml
O-------O Per cent reduction in cell count

TABLE 1 *Effect of Mouse Interferon and the Multiplication Inhibitory and Antiviral Fractions o Multiplication of Osteosarcoma Cells*

Preparation, Antiviral activity		Cell count after 3 da of incubation × 10^3 ±
Control cells		33 3 ± 6 81
Interferon,	400 u/ml	11 6 ± 2 08
Interferon,	10 u/ml	15 6 ± 4 16
AV fraction, (1 10 dilution)	1000 u/ml	23 6 ± 2 10
AV fraction, (1 25 dil)	400 u/ml	28 3 ± 1 53
AV fraction, (1 100 dil)	10 u/ml	29 3 ± 1 58
CMI fraction, (1 25 dil)	1 u/ml	13 0 ± 2 08
CMI fraction, (1 100 dil)	<1 u/ml	22 0 ± 0

) to these findings Although the bulk of CMI ...ity was separated in the low molecular weight ...tions a minor CMI activity was also associated ... the AV fractions in most of our experiments ...s possible that the SDS heat treatment selected ...rferon molecular species with low CMI activity ... the other hand the same treatment separated a ...r molecular weight CMI active component, not ...ssarily part of the interferon molecule.

Another alternative possibility is that the treat...nt caused formation of complexes with CMI ...at SDS alone is cell growth inhibitory Presence ... free SDS in the CMI fraction has been excluded ...tes (4) but it is possible that the employed ...thod did not detect the hypothetic complexes ...ainst this hypothesis argues also the finding of ...mingly identical CMI fractions (6) separated ...m interferon preparation by a different method ...thout presence of SDS

To solve this question it is mandatory that ...ular separation attempts should be done on pure ...terferon when sufficient quantities will be avail...le.

...rts of the experimental work have been carried out at ...e Department of Pediatrics Infectious Diseases Unit ...iversity of Utah Medical Center Salt Lake City ...ah

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show that interferon and the CMI pool dose dependently inhibited the growth of osteosarcoma cells The AV pool had only a minor effect on the cell count Although this effect seems dose dependent the differences were not significant from the controls

In the next experiments the effect of interferon preparation and the CMI fractions on the growth curve of osteosarcoma cells was examined As shown on Fig 2 the suppression by the two agents was comparable From the fourth day of treatment the number of viable cells was significantly reduced by either agents compared to control cultures

The growth inhibitory effect could be visually shown when osteosarcoma cells were grown with various concentrations of interferon preparations or AV or CMI fractions and the cultures were stained after 7 days of incubation Cell growth was clearly suppressed by 50 units of interferon and by comparable dilutions of CMI fractions (1 500) AV fractions had no visible effect on growth

To further delineate the effect of CMI and AV fractions on osteosarcoma cells the uptake of ^{3}H TdR into the cells was determined following treatment with serial dilutions for 24 and 72 hours Serial dilutions of interferon preparation were included for comparizon At the end of the incubation the cultures were pulsed with 1 μCi/well of ^{3}H TdR for 4 hours The data in Fig 3 show that interferon (10 000 to 10 units per ml) and CMI fractions (1 25 to 1 1000 dilutions) dose-dependently reduced the uptake of ^{3}H TdR AV fractions had only a minor influence on the uptake at 1 25 dilution (containing 400 units of interferon per ml) The degree of inhibition was similar after 24 and 72 hours treatment The viable counts were not significantly different after 24 hours After 72 hours the number of cells was dose-dependently reduced by the interferon and by the CMI fractions up to 60 per cent of the controls

DISCUSSION

The osteosarcoma cells from C57Bl/6 mice were established from a tumor produced by irradiation Although the presence of C particles has been shown in these cells the significance of these particles is not known In many respects the murine osteosarcoma seems to be similar to the human osteosarcoma now being treated with interferon with promising results (18) Both systems are highly sensitive to treatment with homologous interferon *in vitro* (11 19) Therefore this model seems to be well fitted for experimental studies with interferon The present findings confirm the sensitivity of murine osteosarcoma cells to the grov inhibitory activity of a homologous interfe preparation after up to 70 passages *in vitro*

A physical separation of antiviral and multiplication inhibitory activities of partially p fied human leukocyte and murine fibroblast int ron preparations has been reported from laboratory earlier (4 6) To confirm these tions we decided to test the active fractions different cell system highly sensitive to multiplication inhibitory activity of Treatment of interferon preparation to CMI activity was performed as reported earlier The CMI and AV activities were demonstrate the same fractions as previously found in L The low molecular weight fractions containing bulk of CMI activity did not inhibit VSV mult cation in L cells However all antiviral fract contained minor quantities of CMI activity

In addition to reducing the cell count the fract containing CMI activity also reduced the incorp tion of ^{3}H TdR into the cells similarly to interfe effect (3) The uptake was not correlated to counts However after 24 hours treatment interferon or CMI fractions the cell count was significantly different from that of control cultu The effect on TdR incorporation is therel probably not only a function of the cell count also inhibition of DNA synthesis or possibl reduction of the transport of the radioac compound through the cell membrane (1 8 Fractions containing AV activity showed onl minor and not significant effect on TdR incorp tion

The interferon preparation employed altho partially purified still contained a high propor of non interferon substances One possible expl tion for the separation of activities as propo earlier (4 6) may be that the interferon mole carries both the AV and the CMI activities and these are separated through the treatment by SD 100 °C This hypothesis may not be true s recently it has been reported that appare homogeneous and pure preparation of m interferon produced in a similar system as o still strongly inhibited cell growth (10)

An alternative explanation may be that the (activity is a contaminant probably prodi together with interferon as a common respons indecers It has been shown (*M Evinger* Nutley J personal communication) that the proportion: of AV and CMI activities in different molec species of apparently pure human leukocyte inte ron may vary considerably Some fractions cor ned low CMI activity together with high activity and vice versa Our data are not contra

y to these findings Although the bulk of CMI vity was separated in the low molecular weight ctions a minor CMI activity was also associated th the AV fractions in most of our experiments is poss ble that the SDS heat treatment selected erferon molecular species with low CMI activity the other hand the same treatment separated a molecular we ght CMI active component, not essarily part of the interferon molecule

Another alternative possibility is that the treat nt caused formation of complexes with CMI nt. SDS alone is cell growth inhibitory Presence [illegible]

gainst this hypothesis argues also the finding of mingly identical CMI fractions (6) separated m interferon preparation by a different method thout presence of SDS

To solve this question it is mandatory that milar separation attempts should be done on pure terferon when sufficient quantities will be avail le.

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rts of the experimental work have been carried out at e Department of Pediatrics Infectious Diseases Unit versity of Utah Med cal Center Salt Lake City ah.

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EXPERIMENTAL ACUTE PYELONEPHRITIS IN GRIVET MONKEYS, PROVOKED BY *STAPHYLOCOCCUS SAPROPHYTICUS*

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Mårdh, P. A., Hovelius, B., Melsen, F. & Møller, B. R. Experimental acute pyelonephritis in grivet monkeys provoked by *Staphylococcus saprophyticus*. Acta path. microbiol. scand. Sect. B, 88: 225–230, 1980.

Urinary tract infection by *Staphylococcus saprophyticus* was provoked in two female grivet monkeys. A non hemagglutinating strain of *S. saprophyticus* was injected into the renal pelvis of one of the animals (monkey I), while in the other (monkey II) a hemagglutinating strain of the same species was inoculated into the bladder by suprapubic puncture. In monkey I, massive hematuria and proteinuria were demonstrated during the post inoculation (p.i.) week, after which the monkey was killed. In monkey II, which was killed after 2 weeks, hematuria and proteinuria were present during the first 5 p.i. days [illegible]

[illegible] were enlarged and there were signs of acute pyelonephritis. The histopathological examination revealed microabscesses, interstitial infiltration and numerous leukocytes in the tubules. Both the ureters of monkey II were congested and microscopically an acute inflammatory reaction was found. Inflammatory signs were also present in the bladder. Scanning electron microscopy revealed cocci adhering to the epithelial lining of the urinary tract.

Key words: *Staphylococcus saprophyticus*; experimental pyelonephritis; grivet monkeys.

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Staphylococcus saprophyticus is a coagulase-negative, novobiocin resistant species, which is a common cause of urinary tract infection (UTI), particularly in young adult women (19, 25).

Most studies on UTI caused by *S. saprophyticus* have focused on the organism as a causative agent of acute, often hemorrhagic, cystitis (11, 21). However, *S. saprophyticus* often seems to infect the upper urinary tract too (5, 10). Patients with this latter type of infection may complain of flank pain and have generally palpatory [illegible] over the kidneys, yet are often only subfebrile. A reduced concentration ability in such patients indicates renal involvement (5, 10).

In rabbits and guinea pigs *Pereira et al.* (20) was able to provoke cystitis by instilling *S. saprophyticus* provided a clot formed in the bladder.

In the present study *S. saprophyticus* was inoculated into the renal pelvis of one female grivet monkey, while in a similar monkey the organism was injected into the bladder by percutaneous suprapubic puncture. Acute pyelonephritis was demonstrated in both monkeys when studied one

and two weeks post-inoculation (p i), respectively. The results of histological, electron microscopic and bacteriological studies are reported

MATERIALS AND METHODS

Animals

Two female grivet monkeys *(Cercopithecus aetiops)*, weighing 1 6 kg and 1 8 kg, respectively, were used The animals, which had been captured in East Africa, were quarantined for 6 weeks, before use

Organisms

One non-hemagglutinating and one hemagglutinating strain of *S saprophyticus*, isolated from urine specimens of two women with signs of acute UTI, were used The strains had been classified as to species according to *Kloos & Schleifer* (9) and the hemagglutinating ability tested as described elsewhere (4) The hemagglutinating strain had been subcultured once after the primary isolation, while the non-hemagglutinating strain had been subcultured several times and stored frozen, prior to use The bacteria were incubated overnight in tryptone broth at 37 °C, washed twice in 0 9% saline and resuspended to approximately 10^{10} bacteria/ml

Experimental Infections

In monkey I, an incision was made through the abdominal wall in the right flank, 2 cm below the costal angle, and the right kidney and renal pelvis were localized 0 2 ml of the suspension of the non-hemagglutinating strain was injected into the lumen of the right renal pelvis The abdominal wall was then closed by sutures in three layers and the wound covered with spray-plaster The operation was performed under aseptic conditions and under general anaesthesia as previously described (17) Monkey I was re-operated 7 days p i The kidneys, the ureters, the bladder and the urethra were extirpated *en bloc* after which the animal was killed

In monkey II, 0 2 ml of the suspension of the hemagglutinating strain was inoculated directly into the bladder by percutaneous bladder puncture after the suprapubic skin had been disinfected with a 2% iodine tincture Fourteen days p i the same operative procedure was undertaken as in monkey I

Bacteriological Studies

Before the experimental infection, specimens were collected with a cotton-tipped swab from the back and from the periurethral region of the two monkeys From each site, two specimens were obtained, one of which was transported in a modified Stuart medium and the other in a selective broth medium containing novobiocin (2 µg/ml) and nalidixic acid (150 µg/ml) (6) The specimens were inoculated onto blood agar plates All staphylococcal strains isolated were tested for their susceptibility to novobiocin (3) The novobiocin resistant strains were classified according to *Kloos & Schleifer* (9) In addition, 6 other grivet monkeys were studied in the same way

Before the animals were infected with *S saprop[...]cus*, urine was collected by percutaneous bla[...] puncture The procedure was repeated every day unt[...] monkeys were killed, viz 1 and 2 weeks [...] respectively All urine specimens were cultured on b[...] agar plates The specimens were also studied [...] *Mycoplasma* and *Ureaplasma* as described elsew[...] (16) Furthermore, tests for proteinuria and hemat[...] using Neostix® (Ames), and quantitative bact[...] cultures were made after the specimens had been frozen (−20 °C)

At operation (7 days p i in monkey I and 14 days in monkey II) specimens were collected from the [...] of the bladder, the ureter and the renal pelvis by mea[...] cotton-tipped swabs The specimens were inocul[...] direct onto blood agar plates

All staphylococcal strains isolated from the urine [...] the urinary tract tissue specimens were identified a[...] species, and tested for hemagglutinating ability

Histological Studies

Specimens from the bladder, the ureters and [...] kidneys, including the renal pelvis were fixed in [...] formaldehyde and paraffin embedded Sections 4 [...] thick, were cut and stained with hematoxylin and e[...]



Fig 1 Inflammation of the right kidney of monke[...] extending from the medulla through the cortex (×5[...]

Specimens from the bladder the right ureter and the al pelvis of monkey II were prepared for scanning ctron microscopy (SEM) as described elsewhere (15)

RESULTS

he general condition of monkey I remained affected during the experimental period, while onkey II became fatigued and presented slow meous movements from day 9 p i until it was led on day 14 p i.

On day 7 p i., the right kidney of monkey I was und to be moderately enlarged The right ureter as swollen and congested, while the bladder, the ft ureter and kidney were macroscopically normal

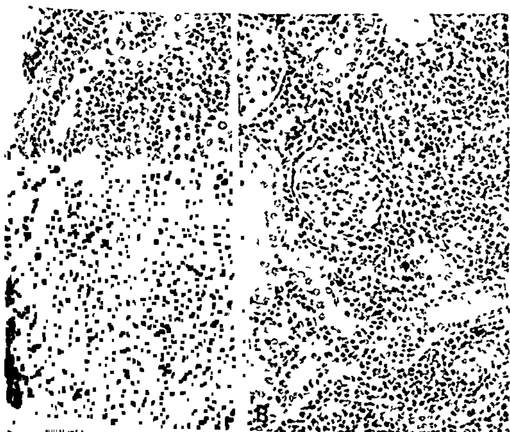
In the right ureter and interstitially in the right dney of monkey I the histological studies revealed n infiltration of polymorphonuclear leukocytes and mphocytes Slight cellular infiltration was seen in e bladder epithelium while the left ureter and the ft kidney were normal

On day 14 p i, both kidneys of monkey II were swollen and congested These alterations were more marked in the right than in the left kidney A small pustule, raised above the surface of the lower pole was detected at the macroscopic examination of the right kidney (Fig 1) The cut surface of the kidney revealed patchy areas of suppuration which were more pronounced in the right kidney Both the ureters were congested along their entire length whereas the bladder did not reveal any gross lesions

Microscopically both kidneys of monkey II showed interstitial infiltration and the tubules were filled with polymorphonuclear leukocytes In the kidneys, microabscesses were observed, more frequently in the right than in the left organ(Fig 2A) There was no glomerular destruction (Fig 2B)

Acute inflammation and cellular infiltration were demonstrated in both the bladder and the ureters (Fig 3)

SEM preparations of the renal pelvis, the ureter and the bladder showed cocci adhering to the epithelial lining There was a non randomized distribution in the adherence – that is cocci (ranging



...tial infiltration with neutrophilic leukocytes and lymphocytes in the tubuli (A) of the right kidney of monkey II. The glomeruli appeared normal (B) (× 100)

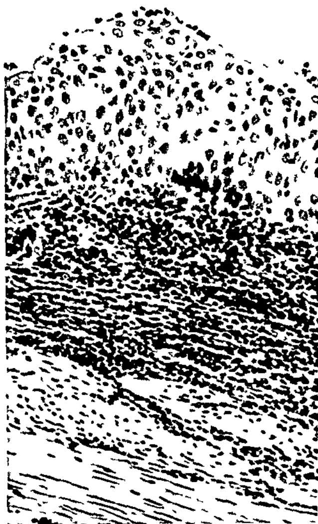


Fig 3 Inflammatory cell reaction mainly with neutrophilic leukocytes and lymphocytes in the bladder wall of monkey II (× 100)

from a few up to 20 bacteria) were attached only to certain epithelial cells

Massive proteinuria and hematuria were found in all urine specimens of monkey I collected after the infection with *S saprophyticus* In monkey II, slight or moderate proteinuria and hematuria were demonstrated during the first 5 days after the injection of *S saprophyticus* but not in the urine specimens obtained on days 6 through 14 p i

periurethral region of both monkeys, as ... from all the 6 additional animals studied Half of the 16 specimens transported in the modified Stuart medium and 11 of 16 specimens transported in the selective broth yielded growth of novobiocin resistant staphylococci Of the altogether 24 novobiocin resistant strains isolated 14 were classified as *S cohnii*, 5 as *S xylosus* 3 as *S saprophyti*... while 2 strains were non typable Monkeys I ... were not among the 3 monkeys from which ... *saprophyticus* was isolated

The urine specimens collected from the monk... before the injection of *S saprophyticus* were ste...

S saprophyticus 10^3–10^4 colony forming u... (CFU)/ml, was isolated from all urine spec... collected p i from monkey I All strains were ... hemagglutinating and their carbohydrate ferme... tion pattern was identical with the strain inject...

In monkey II, all 12 urine specimens coll... p i contained 10^2–10^4 CFU/ml of *S saprophytic*... which were identical as regards hemagglutinat... ability and carbohydrate fermentation pattern ... the strain used to infect the animal

The specimens collected from the bladder and ... right renal pelvis of monkey I on day 7 p i show... growth of *S saprophyticus* while no staphyloc... were isolated from the left renal pelvis In mon... II, *S saprophyticus* was isolated from the bladd... both the ureters, as well as from the left and ri... renal pelvis in the specimens collected on the day ... operation, viz on day 14 p i

In the monkeys, no bacteria except for ... *saprophyticus* were isolated from urine or any ... the specimens collected from the bladder and up... urinary tract

Neither mycoplasmas nor ureaplasmas w... isolated from any of the urine specimens obtai... from monkeys I and II

DISCUSSION

Numerous studies on experimental pyeloneph... have been published (7) In the early experime... rabbits and mice were generally used In th... animals, infection in the kidney is difficult ... establish unless obstruction of the urinary flow ... alterations of the kidney structure are indu... Infection in unaltered kidneys was established ... hematogenous spread of the organism under st... In experiments of the latter kind *Staphylococ... aureus* showed a predilection for the kid... whereas coagulase negative staphylococci prod... localized lesions in the liver, but not in the kid... (18) Later, rats were the usual experime... animals for studies on pyelonephritis as ... condition could be established in this species wi... relatively normal urinary tract (7) To our kno... edge, monkeys have not been used as exper... animals in studies of staphylococcal infections of ... urinary tract

The present investigation shows that *S sa... phyticus* produced an acute interstitial inflamma...

e kidneys in grivet monkeys, in which no ures had been undertaken to interfere with the ry flow and in which no lesions in the urinary had been induced. The lesions were histologi-indistinguishable from acute pyelonephritis in ans In the kidneys of these monkeys, the stital inflammatory cell infiltration was domi-l by polymorphonuclear leukocytes *S saprophyticus* also caused inflammatory changes in the ler and ureters

humans an inflammatory reaction in the rs is a rarely reported complication of UTI nts with UTI caused by *S saprophyticus* tients complain of pain, which at the clinical ination suggests a concrement staggered in the r Whether this pain is due to congestion or mmatory changes in the ureters of such nts is not known

tle is known about pathogenicity factors of ulase negative staphylococci Such factors oc-ng in *S aureus* are lacking in *S saprophyticus* tions such as septicemia and meningitis with *epidermidis* and other coagulase-negative, novo-in-sensitive staphylococci, have generally been ribed to occur in compromised hosts UTI ed by *S saprophyticus* occur, however, in thy women without predisposing disease in the ary tract (5, 11)

n contrast to other staphylococcal species, uding *S aureus* *S saprophyticus* generally es direct hemagglutination of sheep erythrocy 4) The hemagglutinating strain injected into the der provoked more advanced pyelonephritic ges than the non hemagglutinating strain, ch was injected into the renal pelvis In the key infected with the former strain, both eys showed pathological alterations In the key infected with the non hemagglutinating in of *S saprophyticus* pathological changes in kidneys were only found on the injected side hough the animal infected with the former strain killed one week later than that infected with the hemagglutinating strain, the difference observ-might suggest that the hemagglutinin is a viru-e factor In *Proteus mirabilis* a hemagglutinat-factor (type IV pili) seems essential for the blishment of experimental pyelonephritis in rats)

In humans, *S saprophyticus* is known to occur as rinary pathogen only in the urinary tract. In *in ro* experiments, *S saprophyticus* adheres in ater numbers to human urothelial cells than do er established urinary tract pathogens (14), ich might explain the tropism of *S saprophyticus* the urinary tract When studying urothelial cells m the pig, used by *S saprophyticus* is unknown, we did not find any selective adhesive propensity in this bacterium, as compared with *S epidermidis* (2) Urothelial cells from monkeys have not yet been studied in this respect In the two monkeys studied, however, urinary sediments from both had the same microscopic appearance as those from patients with UTI caused by *S saprophyticus*, that is, cocci occurred in clusters and were characteristically attached to cellular elements (2, 5)

In spite of signs of acute pyelonephritis, the urine specimens from the monkeys contained only small numbers of bacteria, viz $< 10^5$ CFU/ml This finding tallies with studies on women with signs of upper UTI caused by *S saprophyticus* (5) *S saprophyticus* has a longer generation time in urine than has *Escherichia coli* (1) Moreover, the cluster-forming tendency of *S saprophyticus* leads to a discrepancy between the true number of bacteria present in the sample and the actual number of CFU demonstrated on cultures of the specimen Freezing of the specimens before culture, as done in this study, also gives a reduced number of bacteria as compared with fresh urine specimens

Coagulase negative, novobiocin-resistant staphylococci occur frequently in the skin flora of a variety of animals In contrast to humans (8) they seem to predominate there over the novobiocin-sensitive species (13) The skin flora of the grivet monkeys does not seem to differ in this respect from domestic and the more commonly used laboratory animals (13), as indicated by the present study

In non human primates various species of *Mycoplasma* can be found, including those recovered from the human genital tract (23) *Mycoplasma hominis* has been isolated from the urine of patients with chronic pyelonephritis (12) The possible part played by this species in the etiology of acute pyelonephritis has recently been considered (24) However, neither *M hominis* nor ureaplasmas were isolated from the two monkeys in our study

S saprophyticus injected into the bladder of rats before and after a bladder diverticulum had been prepared did not invariably cause infection (20) In order to ensure that the inoculated bacteria were not rapidly eliminated from the bladder, *S saprophyticus* was injected together with blood and $CaCl_2$ in order to produce a clot. When using this technique, no pathological alterations were found in the rats, whereas in guinea pigs and rabbits treated in the same way, vascular dilatation, serous exudation and an inflammatory cell reaction in the bladder wall were demonstrated *Staphylococcus epidermidis* produced identical results, although less regularly (20)

Clinical studies (5 10) have indicated that *S saprophyticus* can cause acute pyelonephritis in

man However no histopathological investigation of this condition has yet been reported

The present study has shown that the grivet monkey is suitable to use as an animal model for the investigation of UTI caused by *S saprophyticus* Our study showed that *S saprophyticus* in such animals provoked infection of both the lower and the upper urinary tract leading to cystitis ureteritis and pyelonephritis without any lesions in the urinary tract being produced prior to infection

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This study was supported by grant 512 8215 from the *Danish Medical Research Council* and by grant 16X 4509 from the *Swedish Medical Research Council*

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THE ENDOTOXIN-LIBERATING EFFECT OF ANTIBIOTICS ON MENINGOCOCCI *IN VITRO*

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Andersen, B M & Solberg O The endotoxin liberating effect of antibiotics on meningococci *in vitro* Acta path microbiol scand Sect. B *88* 231–236 1980

Three strains of *Neisseria meningitidis* (two endotoxin liberating and one *in vitro* variant non liberating) were studied during treatment with MIC and 100 times MIC values of benzylpenicillin and chloramphenicol in a chemically-defined protein free medium Treatment with the highest dose of benzylpenicillin had the most rapid effect on meningococci although the antibacterial effect was the same for the two penicillin concentrations after 20 h Chloramphenicol treatment showed a much slower antibacterial effect. After 2 h of antibacterial treatment an increase of filtrable endotoxin in the medium was found for the endotoxin liberating strains only when the highest penicillin dose was used During the same period there was a rapid cell death After 20 hours of treatment, however the endotoxin liberating strains treated with high and low concentrations of penicillin had a markedly reduced content of filtrable endotoxin compared to the controls and to the cultures treated with chloramphenicol Antibacterial treatment had no or only minimal effect on the total content of endotoxin in the cultures compared to the untreated controls The endotoxin non liberating strain had about the same total content of endotoxin as the liberating strains but did not liberate filtrable endotoxin into the medium unless filtered with a much higher pressure through a filter with smaller pore size.

Key words *Neisseria meningitidis* free endotoxin antibiotics

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The morbidity and mortality associated with infections caused by Gram negative bacteria are in many instances considered to be related to the endotoxin of these bacteria. Fulminant meningococcal septicaemia results in release of potent endotoxins which have been shown to produce diffuse vascular damage in many organs (5)

In vitro studies (2 3) have demonstrated that meningococci may liberate endotoxin into the medium during growth and that this liberation of endotoxin varies between strains and differs within the serogroup This difference may perhaps partly explain the various clinical pictures observed in meningococcal diseases

With increasing incidence of meningococci resistant to sulfonamides other antibacterial treatments have been used including the penicillins (1) In addition to sulfonamide resistance the meningococci have shown higher virulence during the last ten years more often causing septicaemia and shock symptoms (1)

The purpose of this investigation is to compare the antibacterial effect of penicillin and chloramphenicol on meningococci grown in a chemically defined protein free medium We have also investigated the short and long lasting effects of the two antibiotics on endotoxin liberating meningococci compared to a non liberating *in vitro* variant

man However no histopathological investigation of this condition has yet been reported

The present study has shown that the grivet monkey is suitable to use as an animal model for the investigation of UTI caused by *S saprophyticus* Our study showed that *S saprophyticus* in such animals provoked infection of both the lower and the upper urinary tract leading to cystitis ureteritus and pyelonephritis without any lesions in the urinary tract being produced prior to infection

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THE ENDOTOXIN-LIBERATING EFFECT OF ANTIBIOTICS ON MENINGOCOCCI *IN VITRO*

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Andersen, B M & Solberg O The endotoxin liberating effect of antibiotics on meningococci *in vitro* Acta path microbiol scand Sect B 88 231-236 1980

Three strains of *Neisseria meningitidis* (two endotoxin liberating and one *in vitro* variant non liberating) were studied during treatment with MIC and 100 times MIC values of benzylpenicillin and chloramphenicol in a chemically-defined protein free medium Treatment with the highest dose of benzylpenicillin had the most rapid effect on meningococci although the antibacterial effect was the same for the two penicillin concentrations after 20 h Chloramphenicol treatment showed a much slower antibacterial effect. After 2 h of antibacterial treatment, an increase of filtrable endotoxin in the medium was found for the endotoxin liberating strains only when the highest penicillin dose was used During the same period there was a rapid cell death After 20 hours of treatment, however the endotoxin liberating strains treated with high and low concentrations of penicillin had a markedly reduced content of filtrable endotoxin compared to the controls and to the cultures treated with chloramphenicol Antibacterial treatment had no or only minimal effect on the total content of endotoxin in the cultures compared to the untreated controls The endotoxin non liberating strain had about the same total content of endotoxin as the liberating strains but did not liberate filtrable endotoxin into the medium unless filtered with a much higher pressure through a filter with smaller pore size

Key words *Neisseria meningitidis* free endotoxin antibiotics

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…eived 12 vii 79 Accepted 8 iii 80

The morbidity and mortality associated with …ections caused by Gram negative bacteria are in …y instances considered to be related to the …doxin of these bacteria. Fulminant meningococ… septicaemia results in release of potent endotox… which have been shown to produce diffuse …cular damage in many organs (5)

In vitro studies (2 3) have demonstrated that …ningococci may liberate endotoxin into the …edium during growth and that this liberation of …dotoxin varies between strains and differs within …e serogroup This difference may perhaps partly …plain the various [illegible] observed in …

With increasing incidence of meningococci resistant to sulfonamides, other antibacterial treatments have been used, including the penicillins (1) In addition to sulfonamide resistance the meningococci have shown higher virulence during the last ten years, more often causing septicaemia and shock symptoms (1)

The purpose of this investigation is to compare the antibacterial effect of penicillin and chloramphenicol on meningococci grown in a chemically-defined protein free medium We have also investigated the short and long lasting effects of the two antibiotics on endotoxin liberating meningococci compared to a non liberating *in vitro* variant

MATERIAL AND METHODS

Bacterial Strains

Three strains were studies two endotoxin-liberating isolates, 270R and 840, and one strain, 840E–, that was a variant from 840 which lost its ability to liberate free, filtrable endotoxin upon subcultivation The strains were serogroups B and typical meningococci in terms of morphology, growth and acid production from sugar, and they were sulfonamide-resistant

Medium

The medium was prepared as described previously (2) 9 867 g dehydrated medium 199 with Earle's unmodified salts without sodium bicarbonate, 3 g dextrose, 0 2 g cocarboxylase and 2 g disodium hydrogenphosphate were dissolved in sterile, pyrogen-free water to 1000 ml The pH was adjusted to 7 5 with 1 M NaOH before filtration through a pyrogen-free prefilter and a membrane filter (2) It was ascertained that all equipment was pyrogen free

Cell Growth

Precultures were grown for 18 h on heated blood agar at 37 °C in 5 per cent CO_2, washed in sterile pyrogen-free saline (0 9% NaCl) three times, and adjusted to an optical density (OD) of 0 6 at 620 nm equivalent to 10^8–10^9 colony-forming units per ml (14)

1 25 ml of each suspension was transferred to 49 ml growth medium The final cultures were grown at 37 °C in 5 per cent CO_2 After 4 h growth, chloramphenicol (Norsk Medisinaldepot, Oslo, 711201) and benzylpenicillin (A/S Apothekernes Laboratorium, Oslo, lot 9/1979) were added to each of two flasks, giving final concentrations of 0 45 µg/ml and 45 µg/ml chloramphenicol and 0 026 µg/ml and 2 6 µg/ml benzylpenicillin The lowest concentration of each antibiotic was equal to or close to, the MIC value of each strain investigated the highest level being appoximately 100 times the MIC value (14)

Viable Counts

Viable counts were done by determining the number of colony-forming units (CFU) (2) CFU plotted in the figures were $\log_{10}$ per ml

Endotoxin Determination

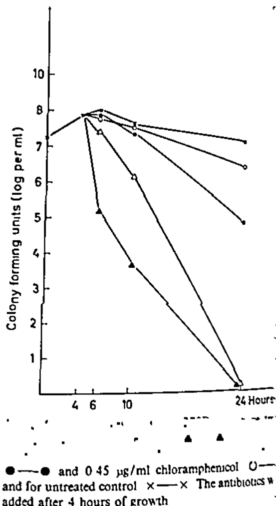
During a growth period of 24 h aliquots of 5 ml were withdrawn from the culture flasks and filtered with pore sizes 0 45 µm and 0 2 µm respectively (Millipore Millex Disposal filter unit Lot no F 8E063040 and Gelman Acrodisc Lot no 11)

Filtrate and unfiltrated samples were stored at −20 °C, or examined immediately

Limulus lysate test was performed as earlier described

Negative and positive controls were always included

Rabbit pyrogen test was carried out according to Pharmacopoea Nordica (11)



●—● and 0 45 µg/ml chloramphenicol O— and for untreated control ×—× The antibiotics w added after 4 hours of growth

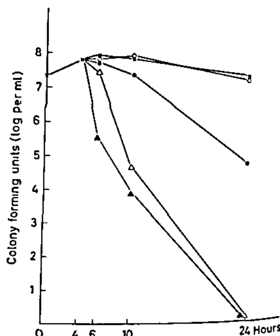


Fig 2 Growth curves for strain 840 during treatmer with 2 6 µg/ml benzylpenicillin ▲—▲, 0 026 µg/m benzylpenicillin △—△, 45 µg/ml chloramphenicol ●—● and 0 45 µg/ml chloramphenicol O—O and for untreated control ×—× The antibiotics wer added after 4 hours of gro

TABLE 1 *Liberation of Meningococcal Endotoxin Through 0 20 µm Compared to 0 45 µm Filters*

Age of culture hours	Strain 270R		Strain 840E−	
	0 20 µm	0 45 µm	0 20 µm	0 45 µm
0	1 1[a]	–	–	–
4	1 1000	1 100	1 100	–
6	1 1000	1 100	1 100	–
10	1 1000	1 1000	1 100	–

Endotoxin dilution in the *Limulus* lysate test. – = endotoxin not detectable

RESULTS

rowth in Medium Containing Antibiotics

The two strains 270R and 840 showed nearly the ame 24 h growth curves The antibiotics had also out the same effect upon the two strains (Fig 1 d 2) The highest penicillin concentration mar-dly reduced the viable count already after 2 h, but ter 20 h both penicillin concentrations gave milar CFU values The two chloramphenicol ncentrations followed the untreated controls ring the first 6 h Thereafter the highest chloram henicol concentration significantly reduced the eningococcal CFU

ect of Pore Size of the Filters on Endotoxin beration

Endotoxin liberation increased when using the allest filter, 0 2 µm (Table 1) A relatively high essure was required to get a filtrate from a cell lture through this filter The strain 840 E− had me endotoxin in the filtrate from the 0 2 µm, but ot from the 0 45 µm filter

When this strain was treated with antibiotics, the ghest concentration of penicillin liberated most dotoxin after 2 h when using the 0 2 µm filter owever when using the 0 45 µm filter no dotoxin was found in the filtrates from the ltures of strain 840 E− treated with antibiotics, in ntrast to the endotoxin liberating strain 270R

E xin Liberation During Influence of Antibiotics

In unfiltered samples, the endotoxin titre rapidly creased during the first 6 h of growth Compared to the controls antibiotics seemed not to influence e total endotoxin content during the first 10 h of owth (Fig 3 a 4 a 5 a) However, some effect, pecially of the penicillins, could be registered after 24 h of growth

The two strains 270 showed nearly the same endotoxin liberation profile and had also the same total content of endotoxin in unfiltered samples during the 24 h period (Fig 3 a and 4 a) When passed through a 0 45 µm filter after 2 h of

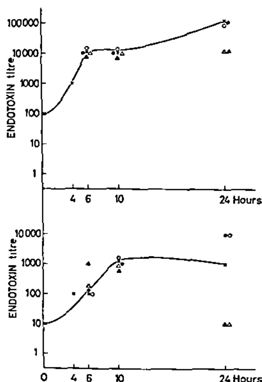
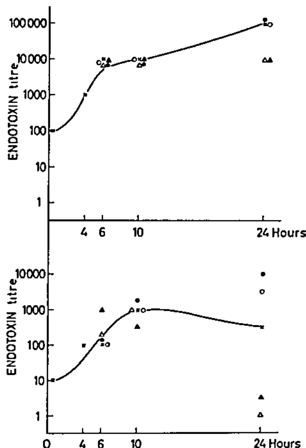


Fig 3 a Endotoxin in unfiltered cultures for strain 270R during treatment with 2 6 µg/ml benzylpenicillin ▲ 0 026 µg/ml benzylpenicillin △ 45 µg/ml chloramphenicol ● and 0 45 µg/ml chloramphenicol O, and for untreated control ×

Fig 3 b As Fig 3 a Endotoxin in filtered samples



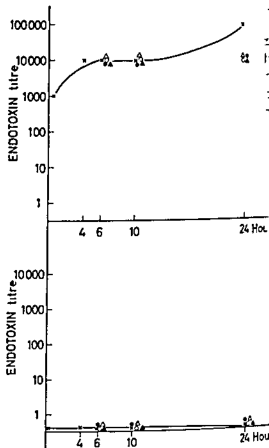


Fig 4 a Endotoxin in unfiltered cultures for strain 840 during treatment with 2 6 µg/ml benzylpenicillin ▲, 0 026 µg/ml benzylpenicillin △, 45 µg/ml chloramphenicol ●, and 0 45 µg/ml chloramphenicol O, and for untreated control ×

Fig 4 b As Fig 4 a Endotoxin in filtered samples

Fig 5 a Endotoxin in unfiltered cultures for s 840E– during treatment with 2 6 µg/ml benzylpenic ▲, 0 026 µg/ml benzylpenicillin △, 45 µ chloramphenicol ●, and 0 45 µg/ml chloramphen O, and for untreated control ×

Fig 5 b As Fig 5 a Endotoxin in filtered samples

antibacterial treatment, the cultures treated with the highest penicillin concentration contained about ten times more filtrable endotoxin than the untreated cultures (Fig 3 b and 4 b) This difference was not found for the cultures treated with the lo penicillin concentration, or with chloramphen Four hours later, the content of filtrable endot decreased in the cultures treated with the hig

TABLE 2 *Endotoxin in Filtrates*[a] *after 2 Hours Antibacterial Treatment of Meningococci* Limulus *Test Compare the Rabbit Pyrogen Test Strain 270R*

Dilution	Control		Penicillin (µg/ml) 0 026		Penicillin (µg/ml) 2 6		Cloramphenicol (µg/ml) 0 45		Cloramphenicol (µg/ml) 45	
	L	R	L	R	L	R	L	R	L	R
1:10	+		+		+		+		+	
1 100	+	–	+	–	+	P	+	–	+	–
1 1000	–		–		+		–		–	
1 10000	–		–		–		–		–	

L = *Limulus* lysate test, R = rabbit pyrogen test, P = pyrogen response, a) (0 45 µm Millipore) + = endoto present, – = no pyrogen response No entry Not done

...llin concentration, while the content increased ... the other cultures

After 20 h of treatment the content of filtrable ...toxin was markedly reduced both for the high ... low penicillin concentrations compared to the ...tes from untreated cultures In comparison the ...tes from the chloramphenicol treated cultures ...ained even more endotoxin than filtrates from ... untreated cultures (Fig 3 b and 4 b) The ...toxin non liberating strain 840E– did not ...rate endotoxin during treatment with antibiotics ...ter the cultures were passed through a 0 45 µm ...ter (Fig 5 b)

The endotoxin content in filtrates from the ...toxin-liberating meningococci was tested also ... rabbits (Table 2) After antibacterial exposition ... 2 h, a pyrogenic response was demonstrated ... with the highest concentration of penicillin (2 6 ...) at a 1 100 dilution.

DISCUSSION

[illegible]

... low concentrations were chosen in this ...stigation, and a rabbit pyrogen test was ...formed as a control

...ect of the Fi...er Pore Si...e on Endotoxin Liberation

It has been shown by others (17) that filters with ... pore size of 0 22 µm are suitable for retaining ...teria, but do not hold back endotoxin In our ...stigation the filter with the smallest pore size ... µm ...

[illegible]

... were handled more gently when a ...ter with larger pores was used As shown earlier ... results using a 0 45 µm filter correspond to ...se obtained in centrifugated samples (3)

...owth During Influence of Antibiotics

Growth curves showed that treatment with ... penicillin at 100 times the MIC value had the ... rapid effect on growing me... [illegible]

Endotoxin Liberation During Treatment with Antibiotics

It has been discussed whether the acute bactericidal effect of penicillin at a high concentration may influence the process of endotoxin liberation (16) Too rapid a destruction of Gram negative organisms during therapy was first noted as a possible danger by *Reilly et al* in 1935 Subsequently others have pointed out the possible connection between antibacterial therapy and endotoxin liberation (7 8) When the meningococci are located mainly in the blood stream high penicillin concentrations may have had a rapid effect on the cell wall In tissues penicillin will usually not reach the peak serum concentration (4)

The three strains of meningococci studied in this investigation all contained abouth the same total content of endotoxin in the unfiltered cultures However when the two endotoxin liberating strains were treated with higher doses of penicillin for 2 h the content of filtrable endotoxin increased During the same period there was a rapid cell death This was not found for the other antibacterial treatments having a rather slower effect on the meningococci

Accordingly when endotoxin liberating meningococci are treated by high doses of penicillin a rapid increase in endotoxin liberation *in vitro* will occur although the total endotoxin content will not change This effect was not found for the meningococcal strain that did not liberation endotoxin unless it was filtered with high pressure through a 0 2 µm filter

If a parallel pattern of endotoxin release upon antibiotic exposure occurs *in vivo* the most severe endotoxin effect may be expected to take place in patients with marked septicaemia when high doses of benzylpenicillin are given intravenously

Another observation was the gradual reduction of the level of free filtrable endotoxin with time for cultures treated by both penicillin concentrations After 20 h of treatment free endotoxin was present only at the lowest dilutions whereas the content of endotoxin of the unfiltered samples was rather high for both penicillin concentrations In contrast the chloramphenicol treated cells seemed to liberate even more endotoxin than the untreated control The explanation may be that penicillin changes peptidoglycan formation in such a manner that the cells liberate endotoxin and also work as endotoxin adsorbers in the culture As shown by others endotoxin may adhere to different membranes both *in vitro* and *in vivo* (6 15) and as earlier shown by us (3) cell wall fragments may form large aggregates of membranes and blebs in the medium

It has been shown for *Esherichia coli* and *Salmonella typhimurium* (13) that lipopolysaccha

ride excretion increases markedly when protein synthesis is stopped by addition of chloramphenicol In our study, the high yield of endotoxin from chloramphenicol-treated meningococci after 20 h of treatment may be explained by a continuing liberation of endotoxin from cells with an intact cell wall that does not work as an absorbant

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a path microbiol scand Sect B 88 237–242 1980

QUANTITATIVE IMMUNOELECTROPHORETIC ANALYSIS OF *SALMONELLA TYPHI* ANTIGENS AND OF CORRESPONDING ANTIBODIES IN HUMAN SERA

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Espersen F Hertz J B Høiby N & Mogensen H Quantitative immunoelectrophoretic analysis of *Salmonella typhi* antigens and of corresponding antibodies in human sera Acta path microbiol scand Sect B 88 237–242 1980

By quantitative immunoelectrophoretic methods 86 different antigens were found in sonicated preparations of a *Salmonella typhi* using corresponding rabbit antiserum Lipopolysaccharide was identified and 5 other antigens characterized Antibodies against a total of 19 *S typhi* antigens were found in human sera The antibody response in patients with typhoid fever was significantly more pronounced compared to that in normal persons When the antibody response was expressed as a precipitin score the predictive value of both a positive (precipitin score ≥ 8) test and a negative test was 100% when sera from patients suffering from typhoid fever were compared with sera from normal persons

Key words *Salmonella typhi* crossed immunoelectrophoresis precipitating antibodies sero-diagnosis typhoid

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The serology of *Salmonella typhi* and other *Enterobacteriaceae* has been extensively studied for several decades The main method used is agglutination of whole bacterial cells but classical immunoelectrophoresis (*Grabar & Williams* (8)) and a few other methods have also been applied (30)

The accumulated knowledge on the serology of *Enterobacteriaceae* concerns mainly O K and H antigens and very useful serotyping schemes have been developed (18 30) However cross reactive enterobacterial antigens have also been studied (17 20)

In most laboratories the antibody response against *Salmonella* infections are measured by means of agglutination of whole bacterial cells (antibodies against O H and Vi antigens Widal reaction) but the diagnostic values of such results are sometimes limited

Later quantitative immunoelectrophoretic methods have been used to investigate the antigenic structure of micro-organisms and to detect precipitating antibodies against a broad spectrum of microbial antigens (2 10 11 15 22) By these methods a hitherto unknown complex antigenic structure of micro-organisms has been revealed (2 5 10 11 12 23 24 25) Furthermore it has been possible simultaneously to investigate the antibody response against a wide range of antigens

The diagnostic values of the antibody response investigated by these methods has been demonstrated in infections caused by *Pseudomonas aeruginosa* and *Candida albicans* (3 4 16)

The purpose of this study therefore is to examine the antigenic structure of *S typhi* and to measure antibodies in serum from normal persons and patients with typhoid fever

MATERIAL AND METHODS

Antigen preparations *Salmonella typhi* (9 12 – – collection number 58) was kindly provided by Drs *I* and *F Ørskov* Statens Seruminstitut Copenhagen This strain was selected because it was superior to other *S typhi* strains investigated in inducing human lymphocyte stimulation and it will be used in current studies of the humoral and cellular immune response in *S typhi* carriers

The bacteria were grown 24 hours on solid medium (Truche) and antigens for electrophoretic experiments (Ref Ag) were prepared by sonication as described in detail previously (12) Colloid concentration was 24 g protein /l (refractometry using human IgG as standard)

Commercial lipopolysaccharide (LPS) from *S typhi* was obtained from Difco Laboratories Detroit USA (*S typhosa* 0901 Westphal)

Heat stable antigens were produced by autoclaving the sonicated preparation (1 hour 120 °C) and surface antigens were identified by absorbing the corresponding rabbit antiserum with whole killed (1% formaline in distilled water 37 °C 1 hour) *S typhi* cells as described previously (14)

Rabbit antisera Antiserum (Ref Ab) was produced by immunizing 5 rabbits intracutaneously with 100 µl of the *S typhi* reference antigen in Freund s incomplete adjuvant according to *Harboe & Ingild* (9) The antiserum was obtained by pooling antisera from successive bleedings after 14 months of immunization The immunoglobulins were isolated and concentrated (9) Before immunization the rabbit sera contained antibodies in low titre against 4 *S typhi* antigens

Rabbit antibodies against common antigen of *Pseudomonas aeruginosa* were prepared as previously described (26)

Human sera 3 groups of human sera were obtained

1) 30 sera from normal persons 15 from blood donors and 15 from persons who had never been immunized with typhoid vaccine or experienced typhoid or paratyphoid fever Median age 27 years (range 3–61 years) 15 male and 15 female

2) 23 sera from 9 patients during typhoid fever Median age 11 years (range 1–33 years) 6 male and 3 female

3) 12 sera from 12 healthy persons who had typhoid fever 2–33 years previously Median age 30 years (range 25–71 years) 3 males and 9 females

Immunoelectrophoretic methods Crossed immunoelectrophoresis crossed immunoelectrophoresis with intermediate gel tandem crossed immunoelectrophoresis and crossed line immunoelectrophoresis were performed on 5 × 5 cm glass plates using Trisbarbital buffer (r/2 = 0 02 pH = 8 6) and 1% agarose from Litex Denmark (Mr = – 0 2) (1 19 20 28)

1 dimension electrophoresis was performed with 1 or 2 µl antigen the intermediate gel contained human sera (20 µl or 40 µl/cm²) bacterial antigen (10 µl or 20 µl/cm²) or saline as a control

Human precipitins were identified by means of the Ag Ab reference pattern and semi quantification was performed by comparing the area of human precipitates with areas of corresponding rabbit precipitat[illegible] standard plates (13) containing increasing amc[illegible] rabbit reference antibodies in the intermediate g[illegible] 0 5 1 2 5 10 20 and 40 µl/cm² In this w[illegible] following titres were established 0 < titre 1 ≤ [illegible] cm² < titre 2 ≤ 0 5 µl/cm² < titre 3 ≤ 1 µl/[illegible] titre 4 ≤ 2 µl/cm² < titre 5 ≤ 5 µl/cm² < tit[illegible] 10 µl/cm² < titre 7 ≤ 20 µl/cm² < titre 8 ≤ [illegible] cm² < titre 9

The precipitin scores were calculated accor[illegible] *Axelsen et al* (4)

Antigens migrating towards the cathode un[illegible] given conditions were analysed by means of c[illegible] immunoelectrophoresis (8)

As protein stain Coomassie brilliant blue wa[illegible] Staining for lipid and polysaccharide was perf[illegible] with Oil Red and Periodic Acid Schiff stain respe[illegible] (27)

Statistical calculations were carried out usi[illegible] Mann Whitney rank sum test and diagnostic spe[illegible] and sensitivity were calculated according to *Wulf[illegible]*

RESULTS

86 antigens were reproducibly detected and e[illegible] rated in the reference *S typhi* Ag Ab pattern [illegible] which migrated towards the cathode Anti[illegible] against these antigens will not be detected wi[illegible] techniques used In Fig 1 the precipitates of t[illegible] anodically migrating *S typhi* antigens are s[illegible] some of these are identified by numbers

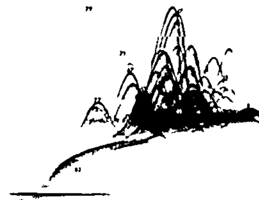


Fig 1 *Salmonella typhi* antigen antibody refe[illegible] pattern Crossed immunoelectrophoresis with interi[illegible] ate gel of 2 µl *S typhi* Ref Ag against Ref Ab (2[illegible] cm²) Saline in the intermediate gel

84 precipitates are seen 16 precipitates are iden[illegible] by numbers

(Technical details First dimension electropho[illegible] anode to the right Second dimension electropho[illegible] anode at the top Staining [illegible] ie brilliant blue

BLE 1 *Characteristic Features of some of the 86 ...ella typhi Antigens Numbers Signify the Antigens in the Reference System*

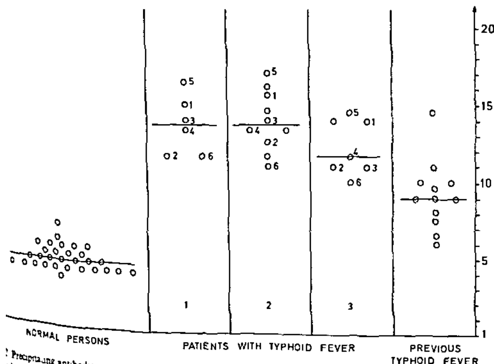
Antigen number ...teristic	9	47	63	67	77	83
...ositive		+	+	+	+	+
...-positive						+
...able						+
...antigen						+
...react with com... LPS						+
...react with com... antigen of ... *aeruginosa*	+					

...le 1 summarizes the characters of some of the ...s. Antigen no 83 is heat stable, a surface ..., PAS-positive, Oil red positive and com... LPS absorbs antibodies against antigen no 83 in crossed line immunoelectrophoresis, and therefore it is identified as Lipopolysaccaride

Monospecific rabbit antibodies against 'common antigen of *P aeruginosa*' precipitated antigen number 9

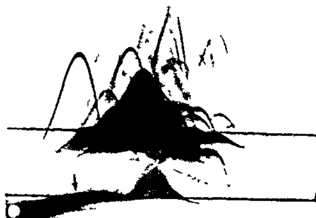
All human sera contained antibodies against antigen numbers 9, 37, 41, 65 and 83, and antibodies against a total of 19 different *S typhi* antigens were found in human sera The numbers of antibody-specificities found in sera from normal persons were in the range 5–7 (median 5), and precipitin scores (Fig 2) were 3½–7 (median 4½)

Both the total number of antibody-specificities (median 9, range 7–11) and precipitin scores (median 13½, range 10–17) were significantly higher in sera from patients than in sera from normal persons ($p < 0.01$) Accordingly the predictive value of both a positive test (i e patient has typhoid fever) and a negative test (i e patient has not typhoid fever) is 100%, if a precipitin score of ≥ 8–10 is chosen as lower limit of a positive test No antibody-specificities were solely detected during infection

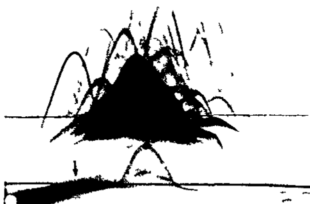


... Precipitating antibodies in sera from normal persons and patients with present or previous typhoid fever The ...dy response is semi-quantified by means of precipitin score paying attention to both the number and titer of ...s (Axelsen et al 1975)
... = after 0–2 weeks infection 2 = after 2–4 weeks and 3 = 4–16 weeks
...zontal line indicates median value
...ential sera from ... are indicated by numbers

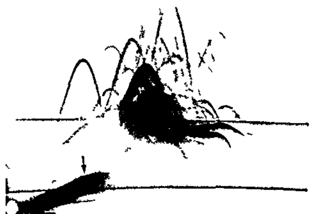
A



B



C



Six of the patients were followed with 3 serum samples one during the first two weeks of infection, one sample during 3rd to 4th week and one sample after 2–3 months (Fig 3) No differences in precipitin scores were found between the first two

Fig 3 Precipitins in consecutive serum sampl one patient with typhoid fever

Crossed immunoelectrophoresis with interme using Ref Ag as antigen and Ref Ab as referen pare with Fig 1)

A Intermediate gel containing serum (40 obtained after 6 days of illnes

B Intermediate gel containing serum (40 obtained after 18 days of illnes

C Intermediate gel containing serum (40 obtained after 70 days Note Titres of precipitir decreased

Precipitins against antigen number 83 and indicated by arrows (compare with Fig 1)

(Technical details as in Fig 1)

samples from individual patients, but a decrease in scores was seen in the last samp

Antigen no 83 and 77 have been found t typhi-specific (7), but no antibodies against were found in any serum investigated

DISCUSSION

A very complex antigenic structure of *S ty* found in agreement with results obtained wi same methods employed in the study of bacterial species (5, 10, 11, 15, 23, 24)

Some of the *S typhi* antigens were charac further Antigen number 83 was lipopolys ride Antigen number 9 corresponds to 'co antigen of *Pseudomonas aeruginosa*', and in dance with this it has been shown to cros with antigens from other gram-negative bacte

In all human sera studied, antibodies aga *typhi* antigens were found The presence of m the antibodies could be explained by the ext cross-reactions between *Salmonella specie* other *Enterobacteriacea* (5, 7), some of whi members of the intestinal flora Antigen nu 83 and 41 were however, not found to cros outside the *Salmonella* group in that collect strains The high prevalence of antibodies a these antigens are therefore more obscure warrents studies on cross reactions with add bacterial species including other *Salmonella* types, as *S typhi* are not endemic in Denma

High precipitin scores were found in sera persons previously suffering from typhoid indicating that a high level of precipitating bodies is maintained for years

Possibilities of using quantitative immunoel phoretic methods as diagnostic aids in clinical are according to *A* ' Observat

...ns found only in patients with infection b) ...er total number of precipitins in sera from ...s during infections c) Increased precipitin ... in sera from patients compared to normal ...s.

...nfection specific precipitins were found and ...was overlapping between normal persons and ...s, when total number of precipitins was used ...promising in this material was the precipitin ...taking both number and titres of precipitins ...onsideration (2) Both the diagnostic sensiti... ...d specificity of this method was 100%, when ...m was to descriminate between normal ...s and patients suffering from typhoid fever ... value of the quantitative immunoelectro... ...c methods as an aid during clinical work ...ere seems promising However, whether it is ...e to discriminate between various *Salmonella* ...ions and infections with other *Enterobacteria*... ...mans to be investigated

...ork was supported by grants from the *Danish ...l Research Council* Mrs *Anni Bethien* is thanked ...d technical assistance The help of Dr *Frits Ør...* ...Statens Seruminstitut, is gratefully acknowledged

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CROSS-REACTIONS BETWEEN *SALMONELLA TYPHI* AND 24 OTHER BACTERIAL SPECIES

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Espersen, F., Høiby, N. & Hertz, J. B. Cross reaction between *Salmonella typhi* and 24 other bacterial species. Acta path. microbiol. scand. Sect. B, 88: 243–248, 1980.

Cross-reactions between antigens from *S. typhi* and 24 other bacterial species were studied by quantitative immunoelectrophoretic methods. A sonicated *S. typhi* antigen preparation and a corresponding pooled rabbit antiserum regularly presenting 86 immunoprecipitates were used as a reference system. Antigens from *Enterobacteriaceae* cross reacted intensively with the *S. typhi* antigens. No cross-reactions between *S. typhi* and antigens from gram positive bacteria were found, and only a few cross-reactions with antigens from gram negative bacteria outside the *Enterobacteriaceae*. The taxonomic perspectives of the immunoelectrophoretic approach are stressed by results showing that the number of cross reacting antigens between *S. typhi* and other *Enterobacteriacea* was positively correlated to the number of identical reactions in a collection of 28 biochemical tests.

Key words: *Salmonella typhi*; crossed immunoelectrophoresis; cross reaction; *Enterobacteriacea*.

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Quantitative immunoelectrophoretic methods have been used to reveal cross reactions between antigens from a wide spectrum of bacterial species (… 8, 11, 17, 18). These methods have a high resolving power with respect to separation of antigens, and they allow a semi-quantitative expression of the degree of cross reactions (8).

The antigenic structure of *Salmonella typhi* as analysed by crossed immunoelectrophoresis has been described in another article (4), which showed that 85 different antigens were detectable in the reference antigen-antibody pattern.

The purpose of the present study was to identify *Salmonella* antigens that are species specific and antigens that are cross reactive. In addition it was attempted to correlate the number of cross reactive antigens to the results of biochemical tests normally used to differentiate *Enterobacteriaceae*.

MATERIAL AND METHODS

Bacteria. The *S. typhi* (9, 12, -, -, collection number 58) reference antigen preparation (Ref. Ag.) and corresponding rabbit antiserum (Ref. Ab.) has been described elsewhere (4).

Preparation of the different bacterial antigens has been described previously, as well as the origin of most of the bacteria recorded in Table 1 (5, 8, 11). The *Campylobacter foetus* kindly provided by Dr. *Mats Walder*, Department of Microbiology, Malmö General Hospital, Sweden, were cultured on solid medium (agar with 5% horse blood) in micro aerophilic atmosphere for 3 days. *Salmonella typhi* (9, 12, Vi, d, –, collection number 59) and *Salmonella typhimurium* (1, 4, 5, 12, i, 1, 2, collection number 10) were kindly provided by Drs. *F.* and *I. Ørskov*, Statens Seruminstitut, Copenhagen. *S. typhi* (9, 12, d, –) was isolated from a blood culture in our laboratory. *S. paratyphi A* (FSK 5789/77) and *S. paratyphi B* (F 3031/77) were kindly provided by Dr. *K. Gaarslev*, Statens Seruminstitut, Copenhagen.

TABLE 1 *Cross-reactions between* Salmonella typhi *(Ref Ag = 86 antigens) and other Bacterial Species The Number Signify the Antigens in the Reference System The Number of Tested Strains and the Group Type or Collection Numbers Are Given in Parentheses*

	Cross-reactive antigens and percentage of cross-reactivity				
	100%	100%–75%	75%–50%	50%–25%	No cross reaction
Salmonella typhi (1) (9, 12 Vi d +)	All other antigens				78
Salmonella typhi (1) (9, 12 d +)	All other antigens				63, 78, 84
Salmonella typhimurium (1) (1, 4 5 12 i 1 2)	All other antigens				77, 83, 84
Salmonella paratyphi A (1)	All other antigens				39, 77, 83, 84
Salmonella paratyphi B (1)	All other antigens				56, 77, 83, 84
Escherichia coli (1) (rough)	All other antigens	63		31, 33, 37	8, 12, 39, 41, 77, 78, 83, 84
Escherichia coli (1) (025 H4)	All other antigens	63		31, 33, 37	8, 12, 38, 39, 41, 77, 78, 83, 84
Shigella sonnei (1)	All other antigens	63	77	31, 33, 37	8, 12, 39, 41, 78, 83, 84
Citrobacter intermedius (1)	All other antigens		45	31, 33, 37	8, 12, 17, 39, 41, 58, 63, 66, 77, 78, 79, 83, 84
Enterobacter cloacae (1)	All other antigens		50	31, 33, 37	8, 12, 17, 25, 30, 41, 45, 57, 58, 63, 69, 77, 78, 83, 84
Klebsiella pneumonia (1) (type 35)	All other antigens			37	8, 12, 17, 31, 33, 39, 41, 57, 58, 77, 78, 83, 84
Klebsiella ozaenae (1) (195)				31, 37, 39	8, 12, 17, 27, 29, 33, 41, 77, 78, 83, 84
Proteus mirabilis (1) (17 precipitates not identified)	All other antigens	9		31, 33, 37, 65	1, 2, 3, 4, 5, 6, 7, 8, 11, 12, 17, 39, 41, 46, 47, 63, 66, 67, 69, 76, 78, 80, 82, 83, 84
Yersinea enterocolitica (1) (type 3) (13 precipitates not identified)	All other antigens	9	45	31, 33, 37	1, 2, 3, 4, 5, 6, 7, 8, 12, 17, 39, 40, 41, 63, 69, 76, 77, 78, 83, 84
Serratia marcescens (1) (22 precipitates not identified)	All other antigens	9		31, 33, 37	1, 2, 3, 4, 5, 6, 7, 8, 12, 39, 41, 63, 71, 77, 78, 82, 83, 84
Vibrio colerae (1) (classica Inaba)	1,75		9	46	All other antigens
Pseudomonas aeruginosa (4) (O groups 3 5 6 11)	2,75			9, 46, 67	All other antigens
Campylobacter foetus (1)				9	All other antigens
Staphylococcus aureus (1) (E 1369)					All antigens
Streptococcus pyogenes (1) (group A)					All antigens
Streptococcus faecalis (1)					All antigens
Streptococcus pneumoniae (1) (type 19A)					All antigens
Bacillus cereus var mycoides (1) (ATCC 11778)					All antigens
Haemophilus influenzae (1) (type b)	10,71	9	59	20, 65, 67	All other antigens
Bordetella pertussis (4) (3803 3825, 3843 and 3860)	75,80				
No [illegible]					

The antigens for immunoelectrophoretic experiments ...obtained by sonication as described previously (7 ... Colloid concentrations (refractometry using human ... as standard) of the antigen preparations were 0 5 to ... g per litre

...electrophoretic methods Antigens from the 24 ...ra were compared with Salmonella Ref Ag using ...following series of immunoelectrophoretic experi...ts Each antigen preparation was run against Ref Ab In 1) Crossed immunoelectrophoresis (21) In 2) Tandem-crossed immunoelectrophoresis with Ref Ag (12) and in 3) crossed line immunoelectrophoresis (13) with the antigen in question applicated in an intermediate gel between the first dimension gel containing Ref Ag and the reference gel containing Ref Ab (absorption of antibodies *in situ*) Each series of immunoplates was repeated at least twice using different antigen/antibody ratios to confirm reaction of identity or partial identity between antigens (1 2)



...1 Comparative cross line immunoelectrophoresis evaluating cross reactions between *Salmonella typhi* (Ref Ag) ...antigens from other *E*... ...
D Crossed ...
Same in the ... (20 µl/cm²)
...ermediate ...
...rbed 100 % ... *typhi* antigens have been
...ipitates ha... indicating partial absorption of Ref Ab ... The area of some of the
Intermediate gel containing *Enterobacter cloacae* antigen Antibodies against 65 *S typhi* antigens have been ...rbed 100 % 19 precipitates are seen
Intermediate gel containing *Proteus mirabilis* antigen Antibodies against 37 *S typhi* antigens are absorbed 100 % ...precipitates are seen.
...nical details First dimension electrophoresis anode to the right Second dimension electrophoresis anode at the ... Staining Coomassie brilliant blue)

The degree of cross-reactivity between antigens was estimated by comparing the area enclosed of a given precipitate after absorption of antibodies *in situ*, with four immunoplates containing 100, 75, 50 and 25%, respectively, of the original concentration of Ref Ab, as described previously (6, 8, 11) In this way the degree of cross-reactivity of individual antigens with antigens of Ref Ag can be expressed as no cross reaction, 25–50, 50–75, 75–100 and 100% respectively Cross-reaction to a degree of 25% or less is considered insignificant owing to the analytical variation of the method (9)

The first dimension electrophoresis was run with 2 µl or 1 µl of the antigen preparation in question The intermediate gel of crossed-line immunoelectrophoresis contained 40 µl/cm² or 20 µl/cm² of antigen or saline as control The reference gel contained Ref Ab (20 µl/cm² or 10 µl/cm²) Immunoplates compared were always run simultaneously Technical details of the immunoelectrophoretic experiments are given in another paper (4)

The routine biochemical reaction used in our laboratory to identify *Enterobacteriacea* to the species level were performed according to *Lautrop et al* (14) 28 tests were included in this study Nitrate reduction, motility in semi solid medium, fermentation of glucose, mannitol (acid and gas), lactose rhamnose, arabinose, xylose, maltose, sucrose, adonitol, dulcitol, sorbitol, inositol and salicin, indole production, Voges Proskauer test growth in the presence of KCN, gluconate oxidation, urease reaction, H_2S production, gelatine (Kohn) liquefaction, decarboxylation of arginine, lysine and ornithine, deamination of phenylalanine, utilization of malonate and utilization of citrate as carbon source

When using the results of these tests for calculation, we attached the same importance to each test, without considering its value in the diagnosis of the species *Enterobacteriacea*

Statistical calculations The Spearman Rank corr test was used (significance level = 5%, one tail (19)

RESULTS

The results of the comparison of antigens fr different bacterial species with the *Salmonell* reference system are given in Table 1

Only 5 antigens proved specific for the *S nella* species (no 8, 12, 41, 77 and 83), and of these were specific for *S typhi* (no 77 an

A high degree of cross-reaction between *S* and the other *Enterobacteriacea* was found most of the antigens cross-reacted 100% B of the high number of antigens in the ref pattern, some of the antigens of *Proteus mir Yersinea enterocolitica and Serratia marce* which precipitated Ref Ab could not be iden These antigens either cross-reacted less than or not at all Fig 1, A-D illustrates the reactions with some *Enterobacteriacea* The sults fit quite well with those of *Brown &* who investigated cross-reactions between *dublin* antigens and antigens from other Ente teriacea (3)

Only a low number of cross-reactions with negative bacteria outside the *Enterobacteriace* found, and no cross-reactions were found be antigens from *S typhi* and antigens from positive bacteria

The number of identical results of t biochemical reactions performed and the num

TABLE 2 *Immunochemical and Biochemical Comparison of the Reference* S typhi *with other* Enterobacter

	Number of identical biochemical tests (Total 28)	Number of 100% cross reacting antigens (Total 84)
Salmonella typhi (9, 12 Vi d –)	27	83
Salmonella typhi (9, 12 d –)	25	80
Salmonella paratyphi B	24	80
Salmonella paratyphi A	22	80
Salmonella typhimurium	22	80
Shigella sonnei	22	72
Escherichia coli (ROUGH)	17	72
Escherichia coli (025 H4)	20	71
Klebsiella ozaenae	19	71
Klebsiella pneumoniae	14	70
Citrobacter intermedius	19	67
Enterobacter cloacae	14	65
Yersinea enterocolitica	19	
Serratia marcescens	14	
Proteus mirabilis	16	

6 cross reacting antigens between *S typhi* and *Enterobacteriacea* are shown in Table 2 is a positive correlation between the number ...ical reactions and the number of antigens cross-react 100% ($r_s = 0.86$ $p < 0.01$)

DISCUSSION

...ross-reaction between *S typhi* and gram ...ve bacteria was found This is in accordance a previous investigation (18)

S typhi antigens cross reacted with antigens *P aeruginosa* *Høiby* found 6 *P aeruginosa* ...ns that cross reacted with *S typhi* (8) 3 of the ...hi antigens cross reacted with *Neisseria* ...ds and *Hoff & Høiby* found that 4 *N* ...ds antigens cross reacted with *S typhi* (6) ...inor differences between the cross reactions ...with the different antigen antibody reference ...ns could be due to the different rabbits used

...antigen found in a wide spectrum of bacterial ...s designated Common antigen of *Pseudomo...erug nosa* has been described and characteri...viously (20) We have shown (4) that it ...reacts with antigen number 9 in the *S typhi* ...nce system This antigen cross reacts with ...ns from all the gram negative bacteria investi... (Table 1) in accordance with other reports our laboratory (6 8 11 17)

...close antigenic relationship between *S typhi* ...other *Enterobacteriacea* was found with more ...40 cross reactive antigens The results are ...cterized by the same pattern either no cross ...on or a 100% cross reaction This is in ...dance with previous investigations (3 6 8 11 18), which also shows that the closer the ...mical relationship the higher the number of ...reactions The apparently species specific anti... number 77 and 83 have both been shown to ...tained by polysaccharide stain and antigen ...ber 83 to be identical to Lipopolysaccharide (4) ...indicates that there were none of the pure ...n antigens in the sonicated *S typhi* prepara... which was species-specific

...significant correlation between the number of ...% cross reacting antigens and the number of ...cal biochemical tests was found This could be ...sequence of a correlation between the genotype ...the 2 fenotypic characteristics antigenic struc... and biochemical capabilities The relatedness ...een our reference strain and those bacteria ...ed in this study expressed as number of ...% cross-reacting antigens (Table 1) fits quite ...into the relatedness found between *Enterobacteriacea* species by means of DNA reassociation (16)

The high number of 100% cross reactive anti gens indicates that many proteins from *Enterobacteriacea* have a similar structure Other authors using different serological methods have shown that serological cross reactivity between phylogenic related proteins is still detectable until 28–40% of the aminoacid sequences are substituted Furthermore the number of aminoacid substitutions are correlated to quantitative immunological difference observed when single proteins were studied (15)

Our results show that by using quantitative immunoelectrophoretic methods it is possible to investigate immunological cross reactivity of 86 different antigens This possibility gives these methods potentials as tools for taxonomic work but the taxonomic implications of the present work should be taken with some reservation until the results have been repeated with a number of international collection strains covering each species

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acta path microbiol scand Sect B 88 249–251, 1980

BRIEF REPORT

A POSSIBLE ASSOCIATION BETWEEN CFA (I) FIMBRIAE AND K99 FIMBRIAE ON *ESCHERICHIA COLI* AND BACTERIAL ADHERENCE TO HUMAN LYMPHOCYTES

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Feilberg Jørgensen N H & Heron I A possible association between CFA(I) fimbriae and K99 fimbriae on *Escherichia coli* and bacterial adherence to human lymphocytes Acta path microbiol scand Sect B 88 249–251 1980

We have explored a possible association between *Escherichia coli* binding to human lymphocytes and plasmid coded fimbriae on the bacterial surface *E coli* with or without the plasmid coded fimbriae CFA(I) K99 and K88 were mixed with freshly-drawn human peripheral blood lymphocytes When the lymphocytes were mixed with *E coli* possessing the CFA(I) fimbriae 59% of the lymphocytes bound bacteria onto the surface whereas only 22% of the lymphocytes bound the CFA(I) derivative The lymphocytes bound 53% and 56% of two K9⁺ strains whereas 22% and 8% of the lymphocytes adhered the same strains without the K99 fimbriae Twelve per cent and 7% of lymphocytes bound bacteria when the strain was $K88^+$ or $K88^-$ respectively Likewise a low (8%) adherence to lymphocytes was found when the *E coli* did not possess fimbriae or flagella

Key words *Escherichia coli* plasmid coded fimbriae human peripheral lymphocytes adherence

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Lymphocytes are divided into two major populations B lymphocytes and T lymphocytes The lymphocytes may be further subdivided using a number of different methods which depend on differences in the lymphocyte surface structure It is possible to subdivide lymphocytes by the use of antibodies directed against cell surface antigens, or receptors for the Fc region of IgG or the capacity of binding heterologous erythrocytes *Teodorescu and co-workers* (13) have recently described a new method for the subdivision of lymphocytes by which they used the natural binding of eleven different species of bacteria to the lymphocyte surface to differentiate between different subgroups of lymphocytes Using this technique it has been possible to differentiate between subgroups of human lymphocytes (13) and between different subgroups of *murine* lymphocytes (9) However since *Teodorescu and co workers* did not identify a structure on the bacterial surface responsible for the adherence of the bacteria to the lymphocyte membrane, we thought it worth while to investigate whether fimbriae may be responsible for adherence of *Escherichia coli* to the surface of human lymphocytes

Fimbriae are hair like structure which are found on the surface of various gram negative bacteria *Duguid and co workers* (3) showed as early as 1955 that *E coli* possessing these structures have the ability to adhere to and agglutinate red blood cells which is today the basis of a test for the bacterial possession of fimbriae *E coli* possessing fimbriae may also bind to the surface of other eucaryotic cells (11) some fimbriated strains of *E coli* are thus known to be able to adhere to the surface of the epithelium in the small bowel where the adherence of bacteria is considered an important step in the pathogenesis of bacterial intestinal infection The fimbriae (K88 K99 and CFA(I)) on *E coli* adhering to intestinal epithelium in pigs (K88) (7) calves (K99) (14) and humans (CFA(I)) (4) differ from other fimbriae by their morphology and biochemical structure and by the observation that the genes for the K88 K99 and CFA(I) fimbriae are localized on plasmids Furthermore the bacterial agglutination of erythrocytes is mannose

resistant in contrast to the agglutination mediated by the other so called »common fimbriae« It is thus possible to investigate whether there is an association between plasmid coded fimbriae on *E coli* and bacterial adherence to human lymphocytes, using matched controls (fimbriae negative derivatives of the K88⁺, K99⁺ and CFA(I)⁺ strains) A fimbriae and flagella free strain was also included in the study

Materials and Methods

Bacterial Strains and Growth Conditions

The strains used in this study were *Escherichia coli* H 10407 (078 K80 H11) is a well-characterized strain possessing the colonization factor antigen (CFA(I)) (4) H 10407-P is a derivative of the strain H 10407 which has lost the ability to produce CFA(I) D 1428 (09 K99⁺) and P 235 (S13) (08 K99⁺ H⁻) are strains possessing the K99 antigen whereas the strains D 1429 (09 K99⁻) and P 235 (08 K99⁻ H⁻) are derivatives of these strains without the K99 antigen D 751 (0100⁺ K88⁺) and D 753 (0100⁺ K88⁻) are isogenic strains except for the K88 antigen (1) JE 2217 is a derivative of *E coli* K12 with no fimbriae or flagella (6)

The bacteria were grown overnight in M 9 broth supplemented with 0 3% vitamin free Casamino acids (Difco) and 0 3% glucose at 37 °C

The bacteria were harvested by centrifugation washed twice with PBS containing 0 02% sodium azide (Merck) (PBS-NaN_3 buffer) and suspended in the PBS NaN_3 buffer at a concentration of about 1×10^7 ml or treated overnight with 5% formaldehyde in PBS washed with PBS and suspended in PBS at the same concentration

Determination of Fimbrial Antigens

Slide-agglutination tests were used for the examination of fimbrial antigens (K88 K99 and CFA(I)) before and after the growth of the bacterial cells CFA(I) antiserum was prepared by immunization of rabbits with living agar grown (M9 agar) H 10407 cells and exhaustive adsorption of the resultant serum with both living and heat killed H 10407 P cells K88 and K99 antisera were kindly provided by Dr *Ida Orskov* from the Statens Seruminstitut Copenhagen

Isolation and Separation of Lymphocytes

Peripheral blood (50 ml) from a healthy volunteer was collected by venous puncture in a screw top bottle containing 10 IU Heparin/ml The blood was diluted with 0 9% NaCl to 100 ml and the lymphocytes were separated from the remaining part of the blood using Isopaque-Ficoll gradients (Pharmacia) Lymphocytes were washed twice in TC199 with 1% Human serum albumin and thereafter suspended in the same buffer at a concentration of 10^5/ml

Assay for the Binding of Bacteria to Lymphocytes

The bacteria and lymphocytes were mixed in a ratio of 200 to 1, the sodium azide concentration was adjusted to 0 02%, and the mixture was centrifuged for 5 min at 500 × *g* The supernatant was decanted, and the lymphocytes were re-suspended in the remaining liquid by vigorously shaking and thereafter examined by phase contrast microscopy

TABLE 1 *Bacterial – Lymphocyte Adherer*

Strain	Fimbriae	Percentage of lympl to which *E coli* ad
H 10407	CFA(I)⁺	59 3 ± 1 4
H 10407-P	–	22 0 ± 6 3
D 1428	K99⁺	53 7 ± 10 4
D 1429	–	22 3 ± 8 9
P 235(S13)	K99⁺	56 4 ± 4 5
P 235	–	7 7 ± 3 2
D 751	K88⁺	12 4 ± 5 4
D 753	–	7 2 ± 3 2
JE 2217	–	8 4 ± 4 1

The results are the mean of at least 4 indep reactions with 3 different donors of lymphocyte

Results

Lymphocytes isolated from the venous bl healthy volunteers were assessed for binding different strains of *E coli* The results from sever assays are summarized in Table 1 Cells bindin than 6 bacteria were scored as positive The figure examples of adherence between *E coli* possessi CFA(I) fimbriae and lymphocytes A lym without bacteria is also shown

The adhesion of formalin-treated bacteria to ly cytes was also explored No difference was found results using unfixed or formalin fixed bacteri (data not shown) and results noted in the table ar obtained using unfixed bacterial cells The strain tested for the possession of the fimbrial antigens slide agglutination after harvest of the bacterial ce the strains were found to be in possession of the fimbrial antigens

A mean of 59% of the human peripheral lymph was found to bind bacterial cells to the surface wl *E coli* strain was CFA(I)⁺ Only 22% of the lym

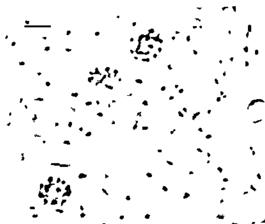


Fig 1 The figure is a photograph using a phase co microscopy showing *Escherichia coli* possessing C pili binding to the lymphocyte surface One non bi lymphocyte is also shown represents 10µ

...were found with bacteria bound to the surface when the CFA(I)⁻ strain was mixed with the lymphocytes ... three per cent and 56% of the lymphocytes were ... with $K99^+$ bacterial cells adhering to the lymphocytes when the experiments were conducted with strains D 1428 and P 235 (S13) respectively When the ... strain D 1429 was mixed with the lymphocytes ... of the lymphocytes bound the bacterial cells ... only 8% of the lymphocytes bound cells from the other $K99^-$ strain P 235 Applying the strain ... the K88 fimbriae to the human lymphocytes did not result in more bacterial cells adhering to the lymphocytes than when using the K88 negative strain ... and 7% respectively) The fimbriae and flagella free strain JE 2217 adhered to about 8% of the lymphocytes

Discussion

The adherence of gram negative bacteria to eukaryotic cells may be mediated through bacterial surface antigenic structures called fimbriae or pili Fimbriae are proteins that can be seen in electron micrographs as fine filamentous structures on the bacterial surface (2) Bacterial adherence to surface epithelium mediated by fimbriae is considered as an important step in the pathogenesis of bacterial infections (5 10 12) Bacterial adherence may however also be used in the identification and classification of lymphocyte subgro... Teodorescu et al (13) have shown as Mayer et al ... that 11 different species of bacteria bind to lymphocytes of human or murine origin They observed bacterial adherence to the lymphocytes from 12% to ... and were able to classify the lymphocytes into groups Some lymphocytes bound bacteria from more than one species but it was possible to classify the lymphocytes on the basis of bacterial binding into subgroups and murine lymphocytes into six groups Further studies with the same group have ... that classification into these subgroups also ... different immunologically functional capabilities

Bacterial binding to eucaryotic cells may be caused by fimbriae. Fimbrial genes may be chromosomal or located on extrachromosomal elements called plasmids Bacterial loss of a plasmid results in an accompany... loss of the plasmid-bound characters an observation that may be used in determining whether a character is plasmid coded but also whether a certain structure takes part in an observed reaction

The human enteropathogen ... E

[illegible]

... was mixed with the ... of these bound bacterial cells to the ... whereas 22% of the lymphocytes adhered the ... strain. The difference in adherence observed ... be caused by the CFA(I) fimbriae on the H 10407 ... or by some unknown character closely connected ... the CFA(I) fimbriae Chromosomally coded fimbriae have been found to be responsible for E coli adhering to kidney cells (12) and these fimbriae may be responsible for the adherence of the CFA(I)⁻ strain to the lymphocytes, because the strain possesses these fimbriae (5) When the experiments were repeated with the bovine enteropathogenic strains possessing the plasmed-coded K99 fimbriae about 55% of the lymphocytes were found with bacteria adhering to the surface whereas only 22% and 8% of the lymphocytes adhered the $K99^-$ strains The observed binding for the $K99^+$ strains to the human lymphocytes could be caused by unknown surface structure but our observations may suggest the existence of a receptor for the K99 fimbriae on the lymphocyte membrane However there seems to be an association between bacterial possession of CFA(I) and K99 fimbriae or some closely related characters and bacterial adherence to human lymphocytes Furthermore the results with the $CFA(I)^+$ and the $K99^+$ strains imply that there may be overlapping in the lymphocyte subgroups to which the bacterial cells bind

The fimbria and flagella free strain JE 2217 and the $K88^+$ and $K88^-$ strains all had a low adherence to the lymphocytes suggesting that these strains did not possess a structure or only minimal amounts of a structure responsible for adherence of these bacteria to lymphocytes

The results of a closer study of the adherence between the enteropathogenic strains and lymphocytes are in preparation

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Drs *Ida Orskov H Williams Smith R Helmuth* and *D G Evans* are thanked for supplying the strains and Dr *Ida Ørskov* is further thanked for the generous gift of K99 and K88 antisera Prof *A Stenderup* is thanked for assistance during the preparation of this paper and Professor *S N Arseculeratne* is thanked for the linguistic revision of the manuscript. Dr *Ursula Friedrich* is thanked for the assistance with the photography Mrs *I Sørensen* is thanked for excellent technical assistance

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Acta path microbiol scand Sect B 88 253–260 1980

AN ANTIGEN COMMON TO A WIDE RANGE OF BACTERIA

2. A Biochemical Study of a Common Antigen from Pseudomonas aeruginosa

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Sompolinsky D Hertz J B Høiby N Jensen K Mansa B Barkholt Pedersen V & Samra Z An antigen common to a wide range of bacteria 2 A biochemical study of a Common Antigen of *Pseudomonas aeruginosa* Acta path microbiol scand Sect B *88* 253–260 1980

Common Antigen (CA) of *Pseudomonas aeruginosa* has been shown to be a protein composed of polypeptide subunits of a molecular weight (MW) of about 62 000 The MW of this protein was estimated to 665 000 by gel filtration on Sepharose CL 6B to 800 000 by electrophoresis on polyacrylamide gradient gels and to about 900 000 by ultracentrifugation on a sucrose gradient By analytical ultracentrifugation with Schlieren optics a sedimentation coefficient ($S_{20°}$ w) of 22 65 was calculated The isoelectrical point was determined to pH 4 4 The antigen was decomposed on exposure to proteolytic enzymes Polysaccharide lipid deoxyribonucleic acid or ribonucleic acid were not demonstrated in CA The amino acid content of CA was determined and no hexosamine or abnormal residues were observed The antigen was degraded when heated to 100 °C for 4 min or when exposed to pH below 4 or above 11 at 4 °C CA has been isolated from the cytoplasmic water-soluble fraction of disintegrated bacteria and only trace amounts could be obtained from envelope fractions after solubilization with Triton X 100

Key Words Common Antigen *Pseudomonas aeruginosa* molecular weight sedimentation analysis amino acid analysis enzyme sensitivity

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Antigens which are shared by a broad spectrum of pathogenic microorganisms are of interest as potential immunogens or diagnostic tools (16) One of the macromolecules found in extracts of *Pseudomonas aeruginosa* seems to be the antigen with the broadest spectrum of cross reactions hitherto reported in bacteria (5 7 8 16) This antigen (Common Antigen or CA) could be obtained in pure form by a combination of salting-out with 18 % (w/v) sodium sulfate and gel filtration on Sephadex G 200 (16) In the present communication some biochemical and biophysical properties of CA are presented

MATERIALS AND METHODS

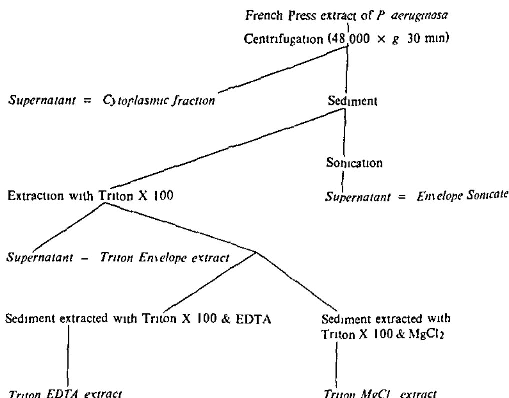
Antigens and Antibodies

Most of the antigens used in the present study (Standard Antigen (St Ag) crude French Press extract and purified Common Antigen) as well as the rabbit immunoglobulins (Standard Antibody (St Ab) anti-CA) have been described previously (16) For this study we

have also prepared antigens from cultures of *P aeruginosa* 888 (16) in fluid Truche medium incubated at 37 °C under constant aeration Part of the culture was harvested in the exponential growth phase (Log phase Ag) and another part in the late declining phase (Lag phase Ag) *We have also examined envelope fractions* from bacteria grown on solid Truche medium (9 16) After disintegration of the bacteria in a French Press (16) remaining whole cells and coars material were removed by centrifugation at 5000 × *g* for 15 min Envelopes and membranes were collected by centrifuga tion at 48000 × *g* for 30 min The supernatant was *designated cytoplasmic fraction The pellet was washed* three times with 10 mM tris (hydroxymethyl) aminome thane HCl (tris) buffer pH 7 0 A portion of 2 5 g wet weight (ww) was suspended in 2 5 ml dist H_2O and sonicated ice cooled for three times 45 seconds (20 000 Hz with a Rapidis 300 W equipped with a 9 5 mm tip) (8 16) The water soluble part was obta centrifugation (48 000 × *g* for 30 min) and des

Envelope sonicate Another portion of 5 g (ww envelope pellet was suspended in 5 ml 10 mM tr containing 2% Triton X 100 pH 7 2 After in overnight at 4 °C the Triton-envelope-extra centrifuged at 48 000 × *g* for 30 min Th obtained was divided in to two portions both c were reextracted one with 2 5 ml 10 mM tr containing 2% Triton X 100 and 1 mM diamine tetraacetate (EDTA) pH 7 2 and the ot 2 5 ml 10 mM tris buffer containing 2% Trito *and 10 mM* $MgCl_2$ *pH 7 2 After incubation o* at 4 °C the supernatants obtained by centri (48 000 × *g* 30 min) were designated Triton extract and Triton $MgCl_2$-extract respectively

The essential steps in the fractionation proce illustrated schematically



Escherichia coli antigens

Antigens from *E coli* were prepared by sonication as used for *P aeruginosa* (16) The bacteria were grown for 48 h at 37 °C on Davis and Mingioli s solidified defined medium (2) either aerobically or strictly anaerobically

diate gel d rocket ere per immunoelectrophoresis with [illegible] formed as previously described (16)

Amino Acid Analysis

Samples for amino acid analysis as well as for other biochemical investigations were obtained by Na_2SO_4 precipitation and gel filtration on Sephadex G 2 were examined for purity by immunochemical and electrophoresis on SDS polyacrylamide gel The amino acid composition was determined Dyrrum model D 500 automatic amino acid an Hydrolysis was carried out 24 h and 72 h in hydrochloric acid with 0 5% phenol in eva ampoules at 110 °C Norleucine was added as an in standard Half cystine was determined as cyste *after oxidation with performic acid (6) and tryptop* described by *Concon* (1)

Polyacrylamide Gel Electrophoresis

The technical details for dodecyl sodium s polyacrylamide electrophoresis (SDS PAGE) were

[illegible] previously (16) Electrophoresis in gels contai[illegible] 2–16% and a 4–30% continuous gradient of [illegible] was used to determine the molecular [illegible] of the native form of CA The electrophoresis [illegible] pH 8 35 contained tris (0 09M) boric acid [illegible] and Na-EDTA (0 003M) Preelectrophoresis performed at 70 V for 30 min Pharmacia sample [illegible] were used allowing a maximal sample size of [illegible] The application was repeated five times into each [illegible] intervals of 30 min to allow the sample to [illegible] into gel by electrophoresis at 70 V Thereafter [illegible]phoresis was continued for 2000 V hours The [illegible] were either used as first-dimension gels in [illegible] with anti-CA in the second dimension agarose gel [illegible] were stained with 0 7% Naphthol Blue Black [illegible] Black) in 7% acetic acid (60 min at room tp) an[illegible] electrophoretically in 7% acetic acid (24V 45–[illegible] A kit of proteins (thyroglobulin 669 × 10^3 [illegible] 440 × 10^3 catalase 232 × 10^3 lactate [illegible] 140 × 10^3 and serum albumin 67 × [illegible] Daltons) was used to calculate the linear relationship [illegible] relative electrophoretic mobility and log of

[illegible] Zonal Centrifugation (17)

[illegible] of 0 5 ml antigen were applied on the top of [illegible] sucrose gradient (10–40% w/v in phosphate [illegible] saline pH 7 38) After centrifugation at 23 000 [illegible] for 1125 min (rotor Spinco SW 50 1) 20 [illegible] of 0 25 ml were collected from the bottom of [illegible]

[illegible] on Sepharose CL 6B

[illegible] 395 × 1 6 cm column (V_t = 180 ml) was [illegible] with a [illegible]

[illegible] determined in fractions of human serum [illegible] 150 × 10^3 apoferritin 440 × 10^3 and [illegible] 669 × 10^3) Fractions of about 1 5 ml [illegible] 6 fractions per hour

[illegible] Coefficient

[illegible] analysis was performed with a Beck[illegible] Model E analytical ultracentrifuge equipped with [illegible] optics and with UV optics An An D rotor at a [illegible] of 60 000 rev/min at 24 °C was used The protein [illegible] in 10 mM tris 100 mM NaCl at pH 7 4 [illegible] coefficient was calculated for water at [illegible]

[illegible] to Lectins

[illegible] of up to 2 mg/ml purified CA were [illegible] with the mannoso- and galactophile lectins of [illegible] and examined for inhibition of hemaggluti[illegible] as described by *Gilboa Garber et al* (4 15) [illegible] to peanut agglutinin and concanavalin A was [illegible] by double gel diffusion in agarose (10) As [illegible] control we used asialofetuin (peanut agglu[illegible] and [illegible] agglutinin (concanavalin A) at the same [illegible] as CA

Isoelectric Focusing of Common Antigen

The isoelectric point (IEP) of Common Antigen was determined by two-dimensional agarose gel electrophoresis of St Ag and purified CA using isoelectric focusing (IEF) in the first dimension and monospecific anti-CA antibody in the second dimension gel IEF was performed on a Pharmacia model FBE 3000 flat bed electrophoresis apparatus at 8–10 °C Agarose of extremely low electroendoosmotic flow (HSIF/B) was kindly donated by Litex Glostrup Cocktails of carrier ampholines® and other technical details were as described for IEF of high molecular weight serum proteins (13) The pH gradient of the IEF gel was determined by suspending pieces of agarose (2 × 0 5 cm) in 0 5 ml double distilled H_2O A pH meter with a microelectrode (Blood Micro System 3MK2 Radiometer Copenhagen) was used Two strips of agarose were cut on both sides of the strip transferred for second dimension electrophoresis The mean of the pH measurement of corresponding pieces was considered as the actual pH value on the IEF strip

Extinction Coefficient at 280 nm

Light adsorption at 280 nm of purified CA in 3 mM tris (pH 7 4) was examined on a Gilford model 2400 S spectrophotometer Protein concentration was determined by the method of *Lowry et al* (11) using bovine serum albumin as a standard The dry weight of CA was determined on lyophilized samples that were dessicated over $CaCl_2$ at 110 °C After cooling the vessels were weighed on a Mettler analytical balance (± 10 µg) by taking the average of three determinations One mg protein correspond to 0 93 mg as measured by dry wt

Degradation of Common Antigen by Hydrolytic Enzymes

Samples of St Ag and purified Ca were incubated with different enzymes and their effect on CA was subsequently analysed by immunochemical methods employing St Ab and anti CA The enzymes used and the conditions of incubation are given in Table 1 In all cases a control analysis of St Ag or CA incubated with the respective buffers without enzyme was included The enzymes were dissolved at a high concentration in a concentrated buffer solution so that the substrate never was diluted by more than 10% After incubation the trypsin digestion was ended by adding aprotinin (20% v/v) Concerning the other enzymes the activity was either stopped by freezing to −20 °C or the immunochemical analyses were carried out immediately after the incubation

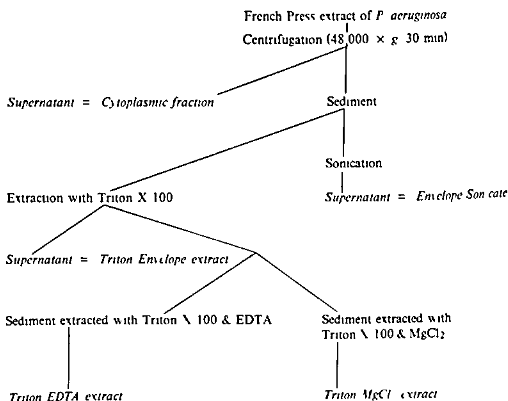
Sensitivity of Common Antigen to Different Hydrogen Ion Concentrations

The crude cytoplasmic extract was dialysed at 4 °C against 2000 volumens of buffer solutions of different pH The stock buffer solution was composed of Citric acid 6 008 g KH_2PO_4 3 893 g boric acid 1 769 g and diethylbarbituric acid 5 266 g in 1 l dist water (3) This solution was mixed with equal volumes of sodium hydroxide of different concentrations to give the pH values indicated under Results After dialysis for 24 h

have also prepared antigens from cultures of *P aeruginosa* 888 (16) in fluid Truche medium incubated at 37 °C under constant aeration Part of the culture was harvested in the exponential growth phase (Log phase Ag) and another part in the late declining phase (Lag phase Ag) We have also examined envelope fractions from bacteria grown on solid Truche medium (9 16) After disintegration of the bacteria in a French Press (16) remaining whole cells and coars material were removed by centrifugation at 5000 × *g* for 15 min Envelopes and membranes were collected by centrifugation at 48000 × *g* for 30 min The supernatant was designated cytoplasmic fraction The pellet was washed three times with 10 mM tris (hydroxymethyl) aminomethane HCl (tris) buffer pH 7 0 A portion of 2 5 g wet weight (ww) was suspended in 2 5 ml dist H_2O and sonicated ice cooled for three times 45 seconds (20 000 Hz with a Rapidis 300 W equipped with a 9 5 mm tip) (8 16) The water soluble part was obta centrifugation (48 000 × *g* for 30 min) and de

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Immunochemical Methods

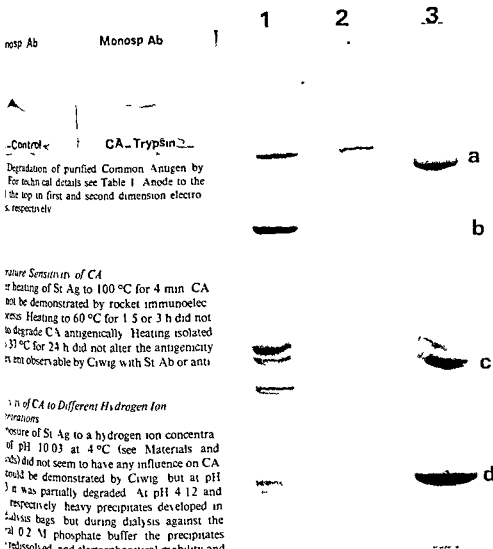
Crossed immunoelectrophoresis with intermediate gel (Ciwig) rocket immunoelectrophoresis and fused rocket immunoelectrophoresis with intermediate gel were performed as previously described (16)

Amino Acid Analysis

Samples for amino acid analysis as well as for other biochemical investigations were obtained by Na_2SO_4 precipitation and gel filtration on Sephadex G 200 were examined for purity by immunochemical crit and electrophoresis on SDS polyacrylamide gels (The amino acid composition was determined wit Dyrrum model D 500 automatic amino acid analy Hydrolysis was carried out 24 h and 72 h in 5 7 hydrochloric acid with 0 5% phenol in evacua ampoules at 110 °C Norleucine was added as an inter standard Half-cystine was determined as cysteic a after oxidation with performic acid (6) and tryptophar described by *Concon* (1)

Polyacrylamide Gel Electrophoresis

The technical details for dodecyl sodium sulf polyacrylamide electrophoresis (SDS PAGE) were gi



Degradation of purified Common Antigen by For technical details see Table 1 Anode to the the top in first and second dimension electro s respectively

rature Sensitivity of CA

r heating of St Ag to 100 °C for 4 min CA not be demonstrated by rocket immunoelec resis Heating to 60 °C for 1 5 or 3 h did not to degrade CA antigenically Heating isolated 37 °C for 24 h did not alter the antigenicity ent observable by Ciwig with St Ab or anti

n of CA to Different Hydrogen Ion ntrations

posure of St Ag to a hydrogen ion concentra of pH 10 03 at 4 °C (see Materials and ods) did not seem to have any influence on CA could be demonstrated by Ciwig but at pH it was partially degraded At pH 4 12 and respectively heavy precipitates developed in dialysis bags but during dialysis against the al 0 2 M phosphate buffer the precipitates redissolved and electrophoretical mobility and city seemed unaltered After dialysis at pH CA was immunologically changed as indica by the faint wollen immunoprecipitates

ular Weight of CA

h SDS PAGE of purified CA after reduction d thiothreitol at 60 °C for 30 min two bands obtained with mobilities corresponding to of 59–62 000 and 62–65 000 Daltons ectively and both were demonstrable by stai g with Coomassie Brilliant Blue (16) On the r hand unreduced CA appeared in the void e of Sephadex G 200 gel filtration indicating MW of > 4–600 000 (16) It was therefore med that the native form of CA was a high ecular weight complex of one or two subunit eptides The following experiments confirmed the untreated form of CA is a protein of a very h molecu

Fig 2 Polyacrylamide electrophoresis of Common Antigen from *Pseudomonas aeruginosa* on a gradient gel A 4–30 % continuous gradient polyacrylamide gel slab was used The protein markers (Track 3) were thyroglobulin (MW 669 × 10^3) apoferritin (440 × 10^3) catalase (232 × 10^3) and lactate dehydrogenase (140 × 10^3) Track 2 Purified Common Antigen Track 1 The supernatant of St Ag after precipitation with 18 % Na_2SO_4 Common Antigen was the first antigen to be withheld by the gel

a Electrophoresis on 2–16 % and 4–30 % Continuous Gradient Polyacrylamide gel slabs

The experiments showed that CA migrated a shorter distance than thyroglobulin (MW 669 × 10^3) By extrapolation on the calibration curve of R_f versus log of MW calculated from the bands of the HMW protein kit a MW of 800 000 was estimated

TABLE 1 *The Influence of Hydrolytic Enzymes on Common Antigen Program of Experiments*

Enzyme	Activity	Solvent (final conc)	Final enzyme conc after mixture with Standard Antigen or Common Antigen	Incubat[illegible] Conditio[illegible]
DNase (Bovine pancreas)	1400 Kunitz units/mg protein	45 mM acetate pH 7 0	400 µg/ml	5 °C 30 m[illegible]
RNase (Bovine pancreas)	100 Kunitz units/mg	5 mM tris 1 mM NaCl pH 7 2	200 µg/ml	37 °C 2
Muramidase (Lysozyme from egg white Sigma grade I)	17 500–30 000 units/mg	30 mM tris 2 mM EDTA pH 7 5–8 0	17 µg/ml	37 °C 2
Trypsin (Bovine pancreas)	Approximately 10 000 BAEE units/mg	10 mM tris pH 7 4	33 µg/ml	35 °C 2
Papain	3 5 mAnson units/mg	75 mM Na phosphate 75 mM NaCl 10 mM cystein 2 mM EDTA pH 7 0	40 µg/ml	35 °C 4

the dialysis bags were transferred to 2000 volumes of 0 2M phosphate buffer pH 7 0 and then to the electrophoresis tris barbital buffer of pH 8 6 2 h in each

Temperature Sensitivity of Common Antigen

St Ag and isolated CA were heated to different temperatures and periodically samples were collected and stored in an ice bath till examination

Chemicals

High molecular weight protein kit (HMW) polyacrylamide gradient gels 2/16 and 4/30 and Sepharose CL 6B were obtained from Pharmacia Fine Chemicals AB Uppsala Sweden All enzymes were products from Sigma Chemical Co St Louis Mo except papain (Merck Darmstadt Germany) Aprotinin (Trasylol 10 000 U/ml) was purchased from Bayer AG Leverkusen Germany Ampholines® were purchased from LKB Produkter AB Bromma Sweden Peanut agglutinin and soybean agglutinin were donated by Prof *N Sharon* The Weizmann Institute of Science Rehovot and Pseudomonas lectins by Dr *N Gilboa Garber* Bar Ilan University Ramat Gan Israel

RESULTS

Growth Phase and Cultivation Conditions

Log phase Ag and Lag phase Ag of *P aeruginosa* were by Ciwig and rocket immunoelectrophoresis with anti CA found to contain similar concentrations of CA The same was the case with antigens prepared from *E coli* cultures on d[illegible] medium with no nitrate (2) cultivated under a[illegible] and strictly anaerobic conditions respectively

The Cellular Localization of Common Antigen

Ciwig of bacterial envelope fractions reve[illegible] considerable amount of CA in the envelope so[illegible] (see p 254) However extraction with Triton released huge amounts of the polysacch[illegible] containing antigen related to the O antigen ([illegible] only very little CA The addition of EDT[illegible] $MgCl_2$ to Triton x 100 had no effect on the r[illegible] of CA This is important since some membrane proteins are released by detergent E[illegible] solvents and some proteins from the inner [illegible] brane are extracted to a greater extent when M[illegible] is added to the detergent (12)

Enzymatic Hydrolysis of CA

Samples of St Ag and purified CA were ex[illegible] to a number of hydrolytic enzymes and then they were examined for degradation by Ciwig monospecific Ab alongside with control sa[illegible] kept in the appropriate buffers at the temperature Deoxyribonuclease ribonuclease muramidase had no effect on CA Trypsin deg[illegible] clearly purified CA (Fig 1) as well as CA in S[illegible] Papain had no clear effect on CA in St Ag caused a strong alteration of the electropho[illegible] mobility of purified CA

[illegible] 1 *Amino Acid Composition of Common Antigen of* Pseudomonas aeruginosa

	Residues per mol of subunits
[illegible]	44
[illegible]	27
[illegible]	27
[illegible]	54
[illegible]	16
[illegible]	57
[illegible]	62
[illegible]	3
[illegible]	47
[illegible]	19
[illegible]	25
[illegible]	42
[illegible]	9
[illegible]	11
[illegible]	5
[illegible]	32
[illegible]	20
[illegible]	4

[illegible] assuming that Common Antigen consists of [illegible] subunits of a molecular weight of 62 000 [illegible] The values are the nearest integers of the mean [illegible] for 24 h and 72 h hydrolyses except where [illegible] indicated

[illegible] for decomposition by extrapolation to zero [illegible] hydrolysis

[illegible] as cysteic acid

[illegible] for 72 h hydrolysis

[illegible] as described by *Concon* (1)

[illegible] Coefficient Isoelectrical Point

[illegible] extinction coefficient of CA at 280 nm for a [illegible] of 10 mg dry wt per ml water $E^{1\%}_{280}$) was [illegible] isoelectrical point (IEP) measured on St Ag [illegible] CA was 4 4 (mean of 4 determinations [illegible] 4 3–4 55)

[illegible] Acid Composition of Common Antigen

[illegible] 2 indicates the amino acid content of CA [illegible] for residues per subunit of MW 62 000 [illegible] one kind of subunit (16) No hexosamine [illegible] residues were observed

DISCUSSION

[illegible] indicated that Common Antigen of [illegible] is a protein It was stained by [illegible] Brilliant Blue in polyacrylamide gels it [illegible] to the proteolytic enzymes papain and [illegible] not to the other [illegible] treatments examined and it reacted with Folin solution of the Lowry reaction (11) Degradation of the antigenic structure by a short heating to 100 °C and sensitivity to very low and very high hydrogen ion concentrations are also properties compatible with a protein The possibility that the molecule is composed of a protein complexed with a non proteinaceous substance cannot be excluded but it should be emphasized that staining for polysaccharide and lipid was negative (9) that binding assays with four lectins gave all negative results that no hexosamine was observed in the amino acid analysis chromatogram that DNase and RNase apparently did not alter its electrophoretic mobility and that dry wt determination and protein determination by the method of *Lowry et al* (11) gave compatible results (see p 255) *CA seems therefore to be a* protein of a MW of 600–900 000 Daltons with a $S_{20°}$ w of 22 65 an $E^{1\%}_{280}$ of 10 26 an IEP of pH 4 4 and it is composed of subunit peptides of about 62 000 Daltons

The molecular weight of CA has been calculated to somewhat different values depending on the techniques used In gradient PAGE it seems clear that thyroglobulin penetrates to smaller gel pores than CA However by gel filtration on Sepharose Cl 6B CA was eluted later than thyroglobulin A possible explanation might be a weak adsorption of CA to the Sepharose gel Also the tertiary structure of CA might be responsible for the discrepancies noted

Ca was demonstrated in and isolated from the water soluble fraction that was obtained when disintegrated bacteria were sedimented at 48 000 × *g* 30 min The growth phase of the bacteria seemed of no influence Extraction of the envelope fraction with the non ionic detergent Triton X 100 released only traces of CA and the addition of EDTA or $MgCl_2$ did not make this extraction method more effective This seems to indicate that no considerable amount of CA remained attached to the bacterial membranes It should however be emphasized that a substantial yield of CA was obtained by sonication of the envelope fraction indeed showing that it contained this substance The failure of Triton X 100-containing solutions to extract CA might indicate that Ca in the envelope fraction is present in the fluid of membrane vesicles and that these are broken by sonication

This study was supported by grants from the *Ministry of Education* Denmark to D S and from the *Danish Medical Research Council* to J B H D S is Established Investigator of the Head Scientist s Bureau Ministry of Health and Efraim Rapoport Professor in Medical Microbiology Bar Ilan University Ramat-Gan The

St-Ab

Monosp Ab

FRACTIONS FROM SEPHAROSE 6B-CL
FIRST FRACTIONS TO THE LEFT

Fig 3 Fused rocket electrophoresis with intermediate gel of fractions from gel filtration on a Sepharose CL 6B column The sample applied to the column was the supernatant of St Ag after precipitation with 18% Na_2SO_4 St Ab was added to the rear gel and anti CA to the intermediate gel

for CA on the 4–30% gel Electrophoresis of the Na_2SO_4 Ag and of isolated Ca did not reveal any proteins of higher MW (Fig 2)

b Gel Filtration on Sepharose Cl 6B

By this method CA was also found to be the most high molecular antigen of the crude French Press extract after precipitation with 18% Na_2SO_4 (Fig 3) The calibration curve was calculated for the relation between elution volume (V_e) and th molecular weight of known proteins By in tion the MW of CA was estimated to 665

c Rate Zonal Centrifugation

Fig 4 shows that CA appeared as the sedimenting substance of Na_2SO_4 Ag Its p in the gradient (fraction 6) was nearly iden that of IgM of normal human serum wher maximum of α_2 macroglobulin was fou fraction 7

Sedimentation Coefficient of Common Antigen

From the Schlieren scan a sedimentation cient $S_{20°}$ w of 22 65 was calculated adsorption optics at 280 nm $S_{20°}$ w was calc to 20 8 but a slight portion of the sedimented slowly corresponding to a $S_{20°}$ w of 5 45 This phenomenon was obtained repe with samples which were apparently pu immunochemical analysis It is possible that S = 5 45 corresponds to the CA subunits (16

Lectin Binding

The possibility that CA is a glycoprotei examined in a number of tests with known le However purified CA did not inhibit hemaggl tion by two Pseudomonas lectins (4 15) ar precipitation was obtained with concanavalin peanut agglutinin in double diffusion test agarose (10)

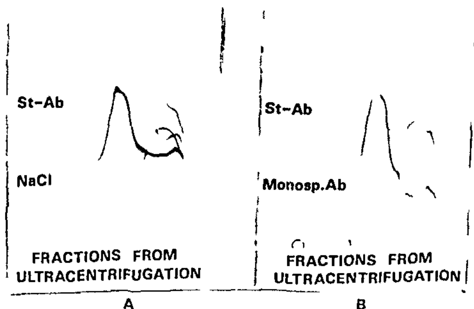


Fig 4 Fused rocket electrophoresis with intermediate gel of fractions of Na_2SO_4 Ag obtained by rate zon centrifugation (10–40% w/v sucrose) The fractions were applied in the same sequence as collected *i e* the highe density to the left Common Antigen seems to be the antigen with the highest density

BIOTYPES OF CAPSULATED AND NON-CAPSULATED *HAEMOPHILUS INFLUENZAE*

Correlation between Biotypes and β-Lactamase Production

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…me C Biotypes of capsulated and non-capsulated *Haemophilus influen ae* Correlation between …es and β-lactamase production Acta path microbiol scand Sect B 88 261–264 1980

Haemoph lus influen ae strains were biotyped according to *Kilian* 393 of the strains were assigned …types *I to V* while 3 strains remained unclassified Eighty nine per cent of the capsulated strains …ed both urease and ornithine decarboxylase biotypes I or IV while 95 per cent of the non …ated strains produced only one of the enzymes biotypes II III or V Of consecutive strains from …per respiratory tract the incidence of β-lactamase positive strains was higher among capsulated …amo… …capsulated β lactamase positive … 15 out of 147 non-capsulated … 1 of biotype IV and 3 of ?

…ords *Haemoph lus influen ae* biotypes β lactamase

…amme Department of Medical Microbiology Solvegatan 23 S 223 62 Lund University of Lund … Sweden

…n submitted 9 … 1980

…the basis of three biochemical characteristics …duction urease activity and *decarboxyla*…tion … *Kilian* recognized five biotypes of … *influen ae* Most capsulated strains …to biotypes I and IV whereas most non …d strains were of biotypes II III and V …deep-seated *H influen ae* infections are in …s caused by capsulated strains and 85– …strains isolated from the upper respiratory …non-capsulated a rough correlation can be …d between biotype and source of infection … 19)

…present investigation 396 *H influen ae* …lated from blood and cerebrospinal fluid …the upper respiratory tract were biotyped …onship between biotype and capsular …on was confirmed Furthermore a correla… …found between β-lactamase production and

MATERIAL AND METHODS

Bacterial Strains

All strains were isolated from routine specimens sent to our laboratory The material included β lactamase producing strains consecutively isolated since 1975 consecutive strains isolated from blood and cerebrospinal fluid since 1976 and consecutive isolates from the upper tract mainly the nasopharynx Only one strain from each patient was included Strains from known close contacts were excluded The strains were preserved at 80 °C in foetal calf serum (Flow Laboratories) with 50 % Tryptone Soya Broth (Oxoid) and 1 % yeast extract (Difco)

Biochemical and Serological Tests

The requirement for haemin and nicotinamide adenine dinucleotide (NAD) was determined by X and V factor discs on agar plates prepared from 20 g Nutrient Broth No 2 (Oxoid) and 7 g Bacto-Agar in 800 ml distilled water Haemolysis was tested on horse blood agar where a streak of *S aureus* served as source of V factor Indole

authors express their gratitude to Mrs *Anni Bethien* Statens Seruminstitut Hvidovre Hospital Denmark Dr *Zvi Kahana* The Weizmann Institute of Science Rehovoth *Uri Karo* Bar Ilan University and *Simon Weiszblum* Tel Aviv University Israel

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TABLE 3 *D stribution of B otypes among Capsulated* Haemophilus influenzae *Strains*

☓	Blood and cerebrospinal fluid[a] β lactamase +	β-lactamase −	Nasopharynx and throat[b] β-lactamase +	β-lactamase −
	3 b	31 b 1 f	10 b	33 b 1 c 12 f
		7 b	1 b	4 b
		1 b	5 e	4 e
	3	40	16	54

secuti e strains

ype b strains (4 β lactamase positive) 1 type c 12 type f and 7 type e (3 β-lactamase positive) were ecutively isolated strains

psulated strains of biotype I These were now ed to biotype II as no decarboxylation of me could be recorded All strains were for porphyrins

he strains included in the material 147 non ated and 56 capsulated strains were consecuti solated from upper respiratory tract speci the capsulated strains comprising 10% of onsecutive isolates Four of the 147 non ated strains (2 7%) and 7 of the 56 capsulated (12 5%) produced β lactamase (χ^2 − 5 78 1025)

DISCUSSION

st bution of biotypes among capsulated and psulated strains was in agreement with that d by other Scandinavian authors (2 13 14) ether 332 capsulated strains biotyped in the t and in the investigations cited 306 were of s I or IV and none of biotypes III or V The ing 26 strains were of biotype II All 15 type ns biotyped hitherto have been assigned to e IV (1 2 4 13 19)

s the capacity to produce capsular polysacc in most *H influenzae* strains parallels the tion of both urease and ornithine decarboxy hereas most non-capsulated strains produce ne of the enzymes

high proportion of biotype II among ated and of biotype I among non-capsulated reported by *Albritton et al* may be due to ological factors or to the inclusion of several s from the same patient (1)

hough there is a correlation between bioche characteristics and virulence i e production of capsular polysaccharide in most strains it would not appear useful to correlate the biotypes strictly with the source of isolation as has been suggested (1 2) Seven of 43 capsulated strains isolated from blood or cerebrospinal fluid were of biotype II in comparison with 5 of 70 capsulated strains isolated from upper respiratory tract specimens (Table 3)

Penicillin resistance in *H influenzae* strains hitherto investigated is attributable exclusively to the presence of the TEM or type IIIa β lactamase The enzyme is determined by transposon A (tn A) often found on plasmids resident in gram negative bacteria (3 6 7 15 21 22)

The incidence of penicillin resistance has been reported as 7–11% among capsulated and 1% among non-capsulated strains which is in agree ment with present results (8 11 17 20) The incidence of β lactamase positive type b strains isolated from blood and cerebrospinal fluid 7 1% (Table 3) may be close to the true incidence in the population as the strains originated from severely ill patients not treated with one or more courses of antibiotics as is often the case in patients with upper respiratory tract infections before a specimen is sent for culture This may be particularly true for capsulated β-lactamase positive strains The signifi cantly higher frequency of β lactamase producers among capsulated than in non-capsulated strains from the upper respiratory tract may therefore be due to the collection of strains from selected patients though it could also indicate that capsula ted strains especially type e have a greater ability to acquire plasmids carrying transposon A than non capsulated strains have

The significant difference in distribution of biotypes bet ulated β-lactamase posi

TABLE 1 *Distribution of Biotypes among 396* Heamophilus influenzae *Strains*

Biotype	Number of strains	Indole production	Urease activity	Ornithin decarboxyl
I	102	+	+	+
II	159	+	+	−
III	93	−	+	−
IV	14	−	+	+
V	25	+	−	+
Non I–V	3			

production was determined in a 24 or 48 h Levinthal broth culture with Ehrlich Bohme reagent Urease activity was determined as described by *Christensen* (5) Decarboxylation of ornithine was determined according to *Møller* (16) Serotyping was done by slide agglutination or gel diffusion (9) β-lactamase was determined by the chromogenic cephalosporin test (10)

The criteria for *H influenzae* were Gram negative rods or coccoid rods with growth requirement for haemin and NAD and no haemolysis on horse blood agar

199 strains were stored prior to biotyping and 197 were biotyped when freshly isolated

153 of the last-mentioned strains were retyped after 6–12 months of storage The indole production was now determined with both Ehrlich Bohme and Kovacs reagents The strains were also tested for biosynthesis of porphyrins from δ-amino-laevulinic acid in order to check the reliability of the X- and V factor disc tests (12)

RESULTS

393 of the 396 strains were assigned to biotypes I to V, while 3 strains remained unclassified (Table 1) 89% of capsulated strains produced both urease and ornithine decarboxylase and were of biotypes I or IV, while 95% of non capsulated strains belonged to biotypes II, III and V – that is, b producing either urease or ornithine decarbo (Table 2)

The distribution of biotypes was signifi uneven for both capsulated (χ^2 = 264 2 4 d f 0 001) and non-capsulated strains (χ^2 = 27 d f, p < 0 001) and also significantly dif between capsulated and non-capsulated strai = 277 0, 4 d f, p < 0 001)

The difference in distribution of β-lacta positive and negative non-capsulated strains, of 133 β-lactamase-positive strains of biotypes IV in contrast to 15 of 147 β-lactamase neg strains, was statistically significant (χ^2 = 12 4 0 001)

Of the 49 strains isolated from bloo cerebrospinal fluid, 43 were capsulated, 42 sero b and 1 serotype f (Table 3) The 6 non capsu strains isolated from blood and cerebrospinal were of biotypes II (4 strains), III (1 strain) a (1 strain) – that is, the same biotypes as recorded for most of the non-capsulated st isolated from the upper respiratory tract

Retyping of 153 strains after 6–12 month preservation gave results identical with the prin typing of freshly isolated strains, except for 2

TABLE 2 *Distribution of Biotypes among Capsulated and Non-capsulated β lactamase Positive and Neg* Haemophilus influenzae *Strains*

Biotype	I	II	III	IV	V	Non I–V	Total
Non capsulated	11	147	93	4	25	3	283
Non-capsulated β + [a]	0	68	45	0	20	1	134
Non-capsulated β − [b]	11	79	48	4	5	2	149
Capsulated	91	12	0	10	0	0	113
Capsulated β +	13	1	0	5	0	0	19
Capsulated β −	78	11	0	5	0	0	94

a) β + = β lactamase positive
b) β − = β lactamase negative

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ANTIBIOTIC SENSITIVITY OF ENTEROPATHOGENIC *ESCHERICHIA COLI* TO MECILLINAM, TRIMETHOPRIM-SULFAMETHOXAZOLE AND OTHER ANTIBIOTICS

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Thoren A Antibiotic sencitivity of enteropathogenic *Escherichia coli* to mecillinam trimethoprim sulfamethoxazole and other antibiotics Acta path microbiol scand Sect B *88* 265–268 1980

Forty-six clinical isolates of enteropathogenic *Escherichia coli* (EPEC) collected in Addis Ababa Ethiopia 1977–1978 were tested for susceptibility to 12 different antibiotics and beta lactamase production Special reference was made to mecillinam and trimethoprim-sulfamethoxazole (TMP SMZ) that were recently shown to be effective in the treatment of severe gastroenteritis caused by EPEC Twenty nine of the strains were of serotype 0111 B4 Thirty of the strains were resistant to 4 antibiotics or more most of these strains belonging to serotype 0111 B4 For mecillinam 19 strains had minimal inhibitory concentration (MIC) ≤0 2 µg/ml 27 strains had MIC 0 8–3 2 µg/ml Regarding TMP SMZ 41 strains had MIC ≤1 µg/ml 5 strains had 2–4 µg/ml No strain was resistant to gentamicin or nalidixic acid Increased production of beta lactamase was correlated to ampicillin resistance

Key words Antibiotic sensitivity enteropathogenic *E coli* mecillinam trimethoprim sulfamethoxazole

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...ed as submitted 17 iv 80

...e use of antibiotics in the treatment of ...hoea caused by enteropathogenic *Escherichia* ... (EPEC) has been controversial especially in ...ic cases of diarrhoea (8) Studies from ...ent geographical areas have advocated their ... (1 2 4 9) and in consequence *in vitro* ...ibility studies of clinical isolates to different ...iotics have been performed (7 10) ... the Ethio-Swedish Pediatric Clinic Addis ...ba, Ethiopia we performed a controlled clinical ... of 49 patients with severe EPEC diarrhoea ... The patients received either mecillinam ...thoprim-sulfamethoxazole (TMP SMZ) or no ...otic treatment The clinical response was very ...rable with a clinical cure rate of 79% for ...nam treatment and 73% for TMP SMZ ...ared to 7% f... controls

diarrhoea caused by EPEC

MATERIALS AND METHODS

Source and Identification of Bacteria

EPEC isolates from 46 patients attending the Ethio-Swedish Pediatric Clinic Addis Ababa Ethiopia during 1977 and 1978 were included Twenty five of the isolates belonged to patients included in the clinical trial previously mentioned All strains were isolated before antibiotic therapy

The bacteria were identified as *Escherichia coli* using the API 20 E system (Analytab New York USA) Serotyping was performed by routine methods as previously described (12) The isolates consisted of the

tive and negative strains may indicate that non capsulated strains producing both ureas and ornithine decarboxylase are unable to acquire plasmids carrying transposon A

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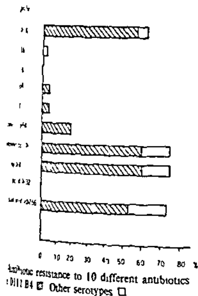
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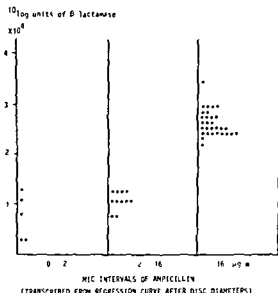
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...ibiotic resistance to 10 different antibiotics
...0111 B4 ▨ Other serotypes □

Fig 5 Beta lactamase activity related to susceptibility of ampicillin

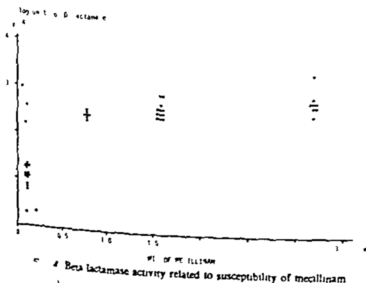
RESULTS

...Testing

...and Fig 2 show the MIC values for ...m and TMP SMZ For mecillinam, only ...0111 B4 had a moderately increased MIC ...the interval of 0 8–3 2 µg/ml for TMP-...ever there was no such obvious correla...IC to serotype One of the multi resistant ...serotype 0111 B4 was re isolated from a ...ter a 5 days course of TMP SMZ The ...e for TMP SMZ had then increased from ...before treatment to 32 µg/ml after ...Fig 3 shows the number of resistant strains to 10 other antibiotics Multi resistant strains generally belong to serotype 0111 B4 Only 2 strains were resistant to kanamycin and neomycin one to cephalexin All strains were sensitive to gentamicin and nalidixic acid

Beta-lactamase Determination

Fig 4 and Fig 5 show the production of beta lactamase related to the susceptibility of mecillinam and ampicillin respectively The 27 strains of serotype 0111 B4 with MIC-values for mecillinam



... 4 Beta lactamase activity related to susceptibility of mecillinam

following serotypes 044 K74 (L) (4), 055 B5 (4), 0111 B4 (29), 0114 K90 (2), 0119 B14 (5), and 0126 B8 (2)

Assay of toxin production on all strains was performed at the National Bacteriological Laboratory, Stockholm, Sweden No strain was toxigenic referring to heatlabile or heatstable toxin production

Sensitivity Testing

The minimum inhibitory concentration (MIC) was determined for mecillinam and TMP-SMZ using the agar dilution method (5) Mecillinam was obtained from Leo Pharmaceutical, Copenhagen, Denmark, and TMP-SMZ from KABI, Sweden The proportion of trimethoprim to sulfamethoxazole was 1 20, thus reflecting the serum ratio (3) *E coli* AT CC 25922 was used as reference strain For all other antibiotics sensitivity testing was performed according to the disc diffusion method (5) PDM medium, that was used for all determinations and antibiotic discs were purchased from Biodisk, Sweden Breakpoints for susceptibility were in accordance to those of the Reference Group for Antibiotic Sensitivity Testing of the Swedish Medical Society's Sections for Medical Microbiology and Infectious Diseases Breakpoints for neomycin were chosen according to Biodisk, Sweden, since neomycin is not yet included in the list of the reference group The breakpoint for mecillinam is still only defined with reference to urinary tract infections while it is not used in this context

Beta-lactamase Determinations

Estimations of beta lactamase activity were kindly performed by *Jens Keiding*, Leo Pharmaceutical Products, Copenhagen, Denmark Estimations were carried out according to a modification of the method described by *Uri et al* (13) Briefly, the chromogenic cephalosporin nitrocephin was used as substrate A change of colour, when the substrate was exposed to suspensions of lactamase producing bacteria was read by v inspection at appropriate intervals One unit of activ defined as the amount of enzyme which in one minu volume of one ml converts the assay mixture : yellow to red

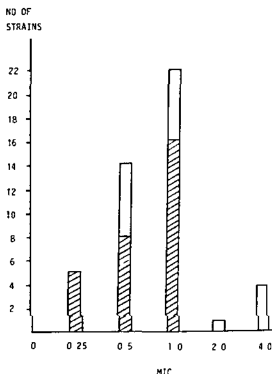


Fig 2 MIC values for TMP/SMZ in weight propor *1 20 Serotype 0111 B4* ▨ *Other serotypes* □

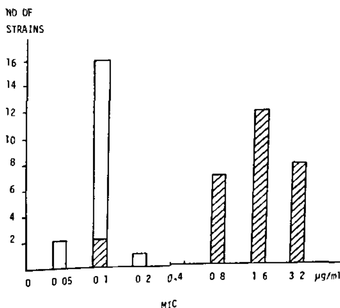
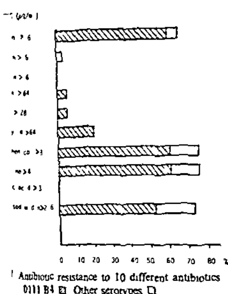


Fig 1 MIC values for mecillinam Serotype 0111 B4 ▨ Other serotypes □



Antibiotic resistance to 10 different antibiotics 0111 B4 ▨ Other serotypes □

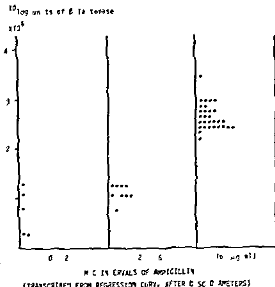


Fig 5 Beta lactamase activity related to susceptibility of ampicillin

RESULTS

...ility Testing

...g 1 and Fig 2 show the MIC values for ...llinam and TMP-SMZ For mecillinam only ...s of 0111 B4 had a moderately increased MIC ... in the interval of 0 8–3 2 µg/ml for TMP ..., however there was no such obvious correla... of MIC ...

...value for TMP-SMZ had then increased from ...g/ml before treatment to 32 µg/ml after ...ment. Fig 3 shows the number of resistant strains to 10 other antibiotics Multi resistant strains generally belong to serotype 0111 B4 Only 2 strains were resistant to kanamycin and neomycin one to cephalexin. All strains were sensitive to gentamicin and nalidixic acid

Beta lactamase Determination

Fig 4 and Fig 5 show the production of beta lactamase related to the susceptibility of mecillinam and ampicillin respectively The 27 strains of serotype 0111 B4 with MIC-values for mecillinam

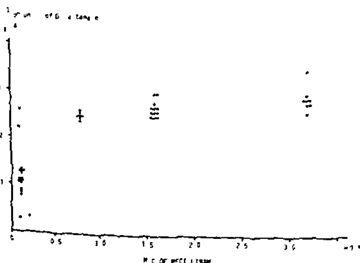


Fig 4 Beta lactamase activity related to susceptibility of mecillinam

following serotypes 044 K74 (L) (4), 055 B5 (4), 0111 B4 (29), 0114 K90 (2), 0119 B14 (5), and 0126 B8 (2)

Assay of toxin production on all strains was performed at the National Bacteriological Laboratory, Stockholm, Sweden No strain was toxigenic referring to heatlabile or heatstable toxin production

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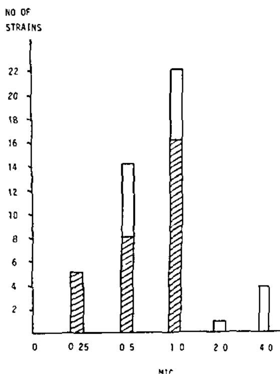


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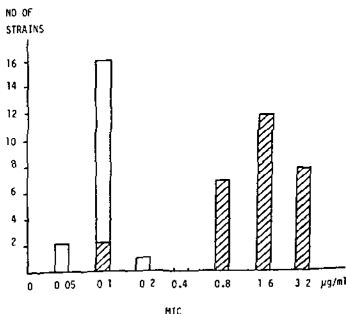
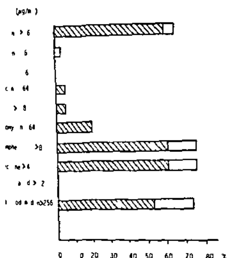


Fig 1 MIC values for mecillinam Serotype 0111 B4 ▨ Other serotypes ☐



3 Antib otic res stance to 10 different antibiotics pe 0111 B4 ▨ Other serotypes ☐

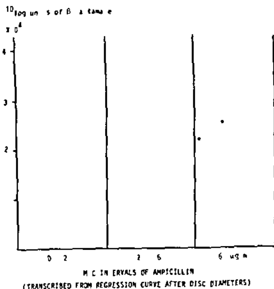


Fig 5 Beta lactamase activity related to susceptibility of ampicillin

RESULTS

S ty Test ng

Fig 1 and Fig 2 show the MIC values for mecillinam and TMP SMZ For mecillinam only strains of 0111 B4 had a moderately increased MIC value in the interval of 0 8 3 2 µg/ml for TMP SMZ however there was no such obvious correlation of MIC to serotype One of the multi resistant strains of serotype 0111 B4 was re isolated from a patient after a 5 days course of TMP SMZ The MIC value for TMP SMZ had then increased from µg/ml before treatment to 32 µg/ml after treatment Fig 3 shows the number of resistant strains to 10 other antibiotics Multi resistant strains generally belong to serotype 0111 B4 Only 2 strains were resistant to kanamycin and neomycin one to cephalexin All strains were sensitive to gentamicin and nalidixic acid

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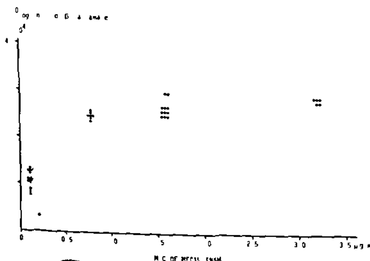


Fig 4 ...mase activity related to susceptibility of mecill nam

in the interval of 0 8–3 2 μg/ml all had increased beta lactamase activity (Fig 4) However 3 strains with high beta lactamase activity had low MIC-values for mecillinam (0 05–0 1 μg/ml) In con trast Fig 5 demonstrates that all strains with high beta-lactamase activity were resistant to ampicillin

DISCUSSION

The treatment of widespread and potentially serious infections such as EPEC gastroenteritis poses some considerations concerning an »ideal« antibiotic high efficacy against the offensive organism low toxi city, low incidence of plasmid mediated R factors and (especially in developing countries) low cost Aminoglycosides and colistin have been widely used as effective drugs for *E coli* infections although they may have some toxic effects Regar ding ampicillin sulfonamides tetracycline and chloramphenicol widespread resistance of EPEC strains has been reported (1 2 4, 7 10)

Mecillinam and TMP SMZ are 2 relatively new drugs reported to have high efficacy against *E coli* infections and low toxicity (3 6) These 2 drugs were successfully used in the treatment of severe EPEC gastroenteritis (12) and *in vitro* investigation of susceptibility confirmed that the MIC values for mecillinam were 5–10 times below the serum concentrations usually obtained with the given dosages of the drug (40 mg per kg of bodyweight i m) (11) A moderate increase of MIC values to mecillinam was always associated with ampicillin resistance and confined to the most frequent serotype (0111 B4) It is not clear whether increased MIC for mecillinam depends on increased beta lactamase activity, since the few ampicillin resistant strains of other serotypes with increased beta lactamase activity as well had low MIC values for mecillinam The susceptibility of these isolates to TMP SMZ was generally good However the marked increase of MIC in one of the strains after treatment with TMP SMZ is an observation

This investigation shows that clinical EPEC isolates from Ethiopia are frequently multi resis tant many of the isolates belonging to serotype 0111 B4 Modern drugs such as mecillinam and TMP SMZ should be considered as alternatives beside established treatment like aminoglycosides

Jens Keiding and *Berit Larsson* are gratefully acknowl edged for technical assistance

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EXPERIMENTAL ENDOCARDITIS IN RABBITS

4 *Experiments with* Serratia marcescens *on the Significance of Serum Susceptibility and Proteolytic Capacity of the Strains and the Influence of an Indwelling Catheter*

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Departments of Diagnostic Bacteriology[1] and Biostatistics[2] Statens Seruminstitut Copenhagen Denmark Science Department[3] St Lawrence College Kingston Ontario Canada and Department of Clinical Immunology and Pathology[4] University of Goteborg Sweden

Gutschik, E Norwood R S Møller S & Olling S Experimental endocarditis in rabbits 4 Experiments with *Serratia marcescens* On the significance of serum susceptibility and proteolytic capacity of the strains and the influence of an indwelling catheter Acta path microbiol scand Sect. B 88 269–276 1980

In order to investigate the course of *Serratia marcescens* endocarditis in groups of rabbits with and without an indwelling catheter 130 rabbits were pretreated to produce left-sided endocarditis Three clinical isolates of *S marcescens* were used to infect the rabbits *i e* CDC O13 (serum sensitive proteolytic) SM 104 (serum resistant proteolytic) and SM 55 (highly serum resistant non proteolytic) Ten rabbits with an indwelling catheter were challenged with CDC O13 and none of them died or showed evidence of endocarditis 28 days later In groups of rabbits with indwelling catheters which were challenged with SM 104 or SM 55 there was a high incidence of endocarditis (19/20 18/20 respectively) while groups without catheters inoculated with the same strains had a lower incidence (5/20 15/20 respectively) In contrast to earlier observations with *Streptococcus faecalis* the clinical and pathological data were not significantly influenced by the presence or absence of proteolytic capacity of the infecting strains The results indicate that the ability of *S marcescens* to establish endocarditis depends significantly on the degree of serum resistance of the strains This difference was only demonstrable in experiments without an indwelling catheter during the infection period The disturbing influence of an indwelling catheter is discussed and it is concluded that experimental models using indwelling catheters are inappropriate for studies on the pathophysiology of endocarditis

Key words Experimental endocarditis rabbits *Serratia marcescens* proteolytic capacity serum bactericidal activity indwelling catheter

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Serratia marcescens is usually considered to be a ...ces with low pathogenicity for man although it ...frequently isolated from the urinary and respira...ry tracts Nevertheless endocarditis caused by *S* ...rcescens has been described with increasing ...quency (5) especially in drug addicts (12)- ...thermore, wound and blood stream infections ...en they do occur, are associated with high death ...es (17 18) The absen... nance of left sided valvular involvement in *Serratia* endocarditis is interesting because it is in contrast to the predominantly right sided location of *Staphylococcus aureus* endocarditis in addicts *Serratia* endocarditis does however in its location resemble the enterococcal endocarditis (3) produced in the rabbit model described earlier (9 10)

The clinical and pathological features and variations in the course of both spontaneous and experimental endocarditis are in part explained by

the influence of one or more bacterial virulence factors such as the ability of the bacteria to adhere to the endocardium (14) their proteolytic capacity (10) and their resistance or sensitivity to bactericidal serum factors (1 4)

In order to learn more about the host parasite relationship in cases of *S marcescens* endocarditis we carried out a series of experiments using our model of experimental rabbit endocarditis (8 9) The aim was to study the course of untreated *S marcescens* endocarditis in cases with and without a retained catheter and after inoculation with strains differing in serum susceptibility and proteolytic capacity

MATERIALS AND METHODS

Strains

Three clinical isolates of *S marcescens* identified by routine laboratory tests and with the following common characters were used Gram negative motile rods indole negative Voges Proskauer positive DNA se positive ornithine and lysine decarboxylase positive and able to reduce nitrate to nitrite They produced acid but no gas in glucose and sucrose and did not ferment lactose The antibiotic susceptibility pattern showed resistance to ampicillin cephalothin polymyxin and chlorampenicol and susceptibility to gentamicin kanamycin and carbenicillin The individual strains were characterized as follows

Strain CDC O13 was non pigmented liquefied gelatin (7) and fibrin (2) within 24 hours and had the serotype designation O13 H_4 (this strain was received by the courtesy of Professor *Walter H Traub* Institut fur Hygiene und Mikrobiologie der Universitat des Saarlandes *Homburg W Germany*)

Strain SM 55 was non pigmented and did not liquefy gelatin or fibrin

Strain SM 104 was pigmented and liquefied gelatin and fibrin within 24 hours

Test for fibrinolysis was carried out on a fibrin plate (2) inoculated with a drop of heavy suspension of bacteria Incubation was at 35 °C and the last reading was recorded after 3 days Liquefaction around the inoculum was evidence of a positive reaction

broth (BHI broth Difco Laboratories) cultures of the strains were grown overnight at 37 °C and then diluted 1 100 in Hanks saline For each strain pairs of tubes each with 0 1 ml rabbit serum were prepared one tube with fresh serum and the other with heat inactivated ... To each tube 0 1 ml of a bacterial ... ted at 37 °C ... d 220 min ... roth chilled at 4 °C was added to each tube ... ring at 4 °C

overnight all tubes were reincubated 4–5 hours and th... agitated and placed directly in a Vitatron photome... The optical density (597 nm) of each tube was recor...

Quantitative blood culture was performed as descr... earlier (10) Blood specimens were taken every ... during the first 2 weeks after infection and three ti... weekly in the following weeks until the animals d... spontaneously or were killed 28 days after chal...

Experimental Groups

Randomly bred male albino rabbits (Ssc CPH) fr... Statens Seruminstitute with an average weight of 300... (SD 123) and an average age of 181 days (SD 30) w... used

Group I Endocarditis was induced in 80 rabbits w... an indwelling catheter during the experimental per... (Table 1)

Group II Endocarditis was induced in 50 rabb... without an indwelling catheter during the experimen... period (Table 1)

Group III Nine healthy non-operated rabbits w... investigated for bacterial clearance after injection ... approximately 10^8 bacteria intravenously Three rabb... were challenged with strains CDC O13 SM 55 and S... 104 respectively From these animals blood samp... were taken for quantitative blood culture 5 s 5 10 ... 20 30 60 and 120 min after challenge and then daily ... 8 days after which the rabbits were sacrificed a... autopsied

Pretreatment for Producing Left sided Sterile Endocarditis

The rabbits were anaesthetized i v with mebun... natrium The average dose at the first operation was 2... mg/kg SD 1 62 (130 observations) and at the seco... operation 23 2 mg/kg SD 2 1 (49 observations) ... polyethylene catheter was inserted into the left ventri... of the heart using the technique described previously (... The catheter was either left *in situ* for 3 days or retain... *for the entire experimental period*

Infection of Pretreated Animals

After 3 days with the catheter *in situ* the rabbits ... experimental group II were subjected to a seco... operation involving the removal of the catheter a... simultaneous injection of 1 ml suspension of *S marcescens* into a marginal ear vein The rabbits ... experimental group I with retained catheter we... challenged in a similar way The bacterial suspension ... saline was prepared from an overnight culture growth ... 5% blood agar This suspension contained approximately 10^8 colony forming units (CFU) per ml (me... $1\ 74 \times 10^8$ range $0\ 74–3\ 67 \times 10^8$)

Autopsy

Autopsy was carried out as described earlier (1... Bacteria recovered from blood vegetations and differe... organs were identified by their characteristic appearan... on blood agar and by microscopy of Gram stained slide... Semiquantitative culture was made from the periton... cavity liver spleen kidneys mesenteric glands an... pleural c...

hearts of five rabbits (two from rabbits infected 1 55 two from rabbits infected with SM 104 all dwelling catheter and one heart from a rabbit with SM 104 but without indwelling catheter) sed exclusively for histological examination as earlier (10) The gross structure of liver mesenteric glands and suprarenal glands was ed infarcts in the kidneys described and counted ir surface extension measured

cal Methods

results from the tests for serum susceptibility of and SM 104 were examined by means of analysis ession, testing the significance of the slopes The curves for SM 55 in fresh and inactivated sera compared using a two-way analysis of variance method was also employed for the comparison of rves showing the in vivo clearance of bacteria mparison between incidence of endocarditis was by Fisher's exact test which was also used for the incidence of early death defined as al time less than 12 days in groups of rabbits with without an indwelling catheter All means given in nt are geometric and the standard deviations are as per cent of the means Weights of spleen heart

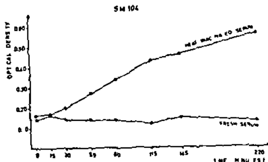
ed vegetations together without taking into account her they died spontaneously or were sacrificed on 8 h day This was the case for the variables weight pleen and heart, but not of vegetations and CFU/g tation.

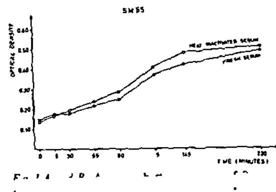
hen the results from rabbits with infected vegetations to SM 55 and SM 104 were compared within each of e two experimental subgroups and the variation ween the two subgroups and between rabbits with and hout infected vegetations was examined

RESULTS

...ty of the Infecting Strains to the Bactericidal ...ct of Normal Serum

In the photometric growth assay (test A) CDC 13 was found to be markedly sensitive to three mal human sera and 20 normal rabbit sera the SM 55 and SM 104 were classified as serum stant. In order to further examine the resistance (SM 55 and SM 104 an assay by bacterial growth serum (test B) was carried out Fig 1 A and B ows clearly that strain SM 55 gave good growth both heat inactivated serum and fresh rabbit um, though significantly weaker in the latter that SM 104 grew only in inactivated serum ring the 220 min incubation period These results monstrate that SM 55 was significantly more rum resistant than SM 104





different time periods as indicated

Clearance of the Different Strains of S marcescens in Non treated Rabbits

Fig 2 shows that after i v challenge of rabbits the elimination of the organisms was most rapid for the serum sensitive strain CDC O13 and that the moderately serum resistant strain SM 104 was cleared more rapidly than the highly serum resistant

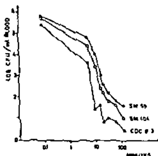


Fig 2 Bacterial clearance of *Serratia marcescens* strains Three rabbits were challenged i v (1 ml of a suspension of approximately 10^8 bacteria/ml range 1 25–1 77 × 10^8) with strains SM 55 SM 104 and CDC O13 respectively Blood samples were taken after 5 seconds 5 10 15 20 30 60 and 120 min (log scale) as indicated

TABLE 1 *Incidence (Ratio Infected Challenged Rabbits) of Experimental* Serratia marcescens *Endocarditis in Rab at Indicated Time after Challenge*

Rabbit group	Infecting strain	Rabbits sacrificed after			Rabbits dead spontaneously before day 28	Rabbits sacrificed after day 28	Total
		1 day	2 days	3 days			
I	SM 55	5/5	5/5	4/5	12/12	6/8	18/20
Catheter	SM 104	5/5	5/5	5/5	15/15	4/5	19/20
in situ	CDC O13	ND	ND	ND	None	0/10	0/10
II	SM 55	3/5	ND	ND	11/11	4/9	15/20
Catheter removed	SM 104	3/5	ND	ND	4/4	1/16	5/20

ND = not done

strain SM 55 The significant differences are apparent after 5 min and then persist practically unaltered during the 120 min observation period

Incidence of Endocarditis Related to Presence or Absence of Catheter and Characteristics of the Infecting Strains

Left sided sterile endocarditis was induced in 130 rabbits The establishment of bacterial endocarditis after challenge was confirmed by isolation of *S marcescens* from the vegetations or by histological examination In group I a high incidence of bacterial endocarditis was recorded in rabbits infected with the highly serum resistant strain SM 55 and with the moderately serum resistant strain SM 104 while none of the rabbits challenged with the serum sensitive strain CDC O13 developed infection ($p<0.001$) (Table 1) In group II the incidence of bacterial endocarditis was 3 out of 5 due to both SM 55 and SM 104 one day after challenge Howe in rabbits observed for 28 days a significantly lo incidence ($p = 0.004$) of bacterial endocarditis oc red due to the moderately serum resistant strain 104 than due to the more resistant strain SM The different incidence of endocarditis due to 104 in groups I and II is highly signifi ($p<0.001$), whereas the incidence of SM infections was not significantly different in the groups

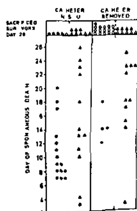


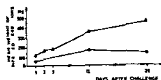
Fig 3 Survival time of rabbits with *Serratia marcescens* endocarditis Each dot represents one rabbit inoculated with SM 104 (● infected vegetations O non infected vegetations) or with SM 55 (▲ infected vegetations △ non infected vegetations)

Survival Time Related to Presence or Absence Catheter

Fig 3 shows the survival times of rabbits died spontaneously of endocarditis and the num of surviving rabbits sacrificed 28 days a challenge The number of early deaths (*i e* surv time <12 days) is significantly higher in rab with an indwelling catheter (14/27) than in th lacking a catheter (1/15) ($p<0.001$) Most of rabbits with an indwelling catheter which survi for 28 days still had infected vegetations w most of those without a retained catheter had ste vegetations or macroscopically normal valve l lets This is particularly true for rabbits challen with the moderately serum resistant strain SM 1

Appearance Weight and Bacteriology of Vegetati

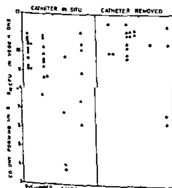
Distribution of vegetations was different rabbits with and without an indwelling cathe during the experimental period Rabbits withou catheter had their vegetations chiefly on the ao valve leaflets, while rabbits with an indwell catheter also had large vegetations outside the ao valve leaflets, *i e* on the wall of the aorta and alo the catheter on the wall of the left ventricle It wa regular feature that only one or two of the th aortic valve leaflets was affected and that vegetations rema ited to the origina



g 4 Mean weight (mg) of infected vegetations in bbits succumbed spontaneously or sacrificed at the me indicated The results for the non sacrificed groups re plotted against the mean survival time 12 4 days △ bbits with an indwelling catheter O rabbits without n indwelling catheter

fected area. No dissimilarities were observed in he consistency or friability of vegetations infected vith different strains Visible destruction of valve eaflets was not recorded

Vegetations were found in all rabbits with an ndwelling catheter but only in 23/50 of those ithout All rabbits challenged with the serum ensitive strain CDC O13 had sterile vegetations mean weight 76 2 mg (SD 89%) The weight of nfected vegetations was found to be significantly igher in rabbits with an indwelling catheter at all ints during the post-exposure period than in bbits where the catheter had been removed (Fig 4) There was no significant difference between the weights of vegetations in rabbits infected with SM 5 and in those infected with SM 104 The bacterial ensity in the vegetations of rabbits that sponta eously succumbed to endocarditis was high The ean number of CFU in rabbits with an indwelling atheter was $10^{9.9}$/g vegetation and in those ithout it was $10^{10.7}$/g The level of CFU in the egetations was not significantly different in rabbits



g 5 B... km in ... kts de ... ndocar ... ulet s

inoculated with SM 55 and with SM 104 Surviving rabbits sacrificed on day 28 of the experimental period showed considerable variation in the number of CFU indicating different stages in the infectious process (Fig 5) The mean number of CFU/g vegetation on days 1, 2 and 3 after challenge was $10^{6.75}$ $10^{8.77}$ and $10^{9.00}$, respectively in rabbits with an indwelling catheter, indicating the rapid development of the infection In rabbits without an indwelling catheter a result of $10^{4.98}$ CFU/g vegetations was recorded on day 1 after challenge *i e* significantly different ($p<0.005$) from the level of CFU in rabbits with an indwelling catheter

Histopathology of Vegetations

The vegetations consisted of an amorphous eosinophilic mass with dispersed single granulocytes mononuclear cells and aggregates of bacteria occurring as oval or circular rings The density of bacteria observed was very high in all vegetations on the aortic valve leaflets From vegetations outside the aortic valves the foci of bacteria were small or lacking and the tendency towards endothelialization was usually most pronounced here No clear-cut histological differences could be observed between vegetations infected with the proteolytic strain SM 104 and the non proteolytic strain SM 55

Quantitative Blood Culture

The nine non-operated rabbits in group III were observed for circulating bacteria in the blood stream 1 to 9 days after challenge by taking daily blood specimens None of the rabbits inoculated with the serum sensitive strain CDC O13 had positive blood cultures In the majority of rabbits inoculated with SM 55 and SM 104 positive blood cultures but only few bacteria per ml were observed during the first 4 days after challenge but not later

From the rabbits pretreated for endocarditis 47 3% of 1354 blood specimens were bacteriologically positive None of the rabbits inoculated with CDC O13 had positive blood cultures during the 28 days of the experimental period In rabbits with an indwelling catheter inoculated with SM 55 or SM 104 positive blood samples were recorded in 51 6% (261/506) while in catheter free rabbits only 38 2% (230/608) were positive Furthermore rabbits with an indwelling catheter that died spontaneously of endocarditis had more positive blood cultures (197/290 ~ 67 9%) than those without a catheter (170/287 ~ 59 2%) Significant differences could not be demonstrated between groups of rabbits inoculated with SM 55 and SM 104

The level of CFU/ml blood was between 10 and 100 in the majority of positive blood cultures High numbers of bacteria *i e* at the level of 10^3 or 10^4/

TABLE 1 *Incidence (Ratio Infected Challenged Rabbits) of Experimental* Serratia marcescens *Endocarditis in Ra*
at Indicated Time after Challenge

Rabbit group	Infecting strain	Rabbits sacrificed after			Rabbits dead spontaneously before day 28	Rabbits sacrificed after day 28	Total
		1 day	2 days	3 days			
I	SM 55	5/5	5/5	4/5	12/12	6/8	18/20
Catheter	SM 104	5/5	5/5	5/5	15/15	4/5	19/20
in situ	CDC O13	ND	ND	ND	None	0/10	0/10
II	SM 55	3/5	ND	ND	11/11	4/9	15/20
Catheter removed	SM 104	3/5	ND	ND	4/4	1/16	5/20

ND = not done

strain SM 55 The significant differences are apparent after 5 min and then persist practically unaltered during the 120 min observation period

Incidence of Endocarditis Related to Presence or Absence of Catheter and Characteristics of the Infecting Strains

Left-sided sterile endocarditis was induced in 130 rabbits The establishment of bacterial endocarditis after challenge was confirmed by isolation of *S marcescens* from the vegetations or by histological examination In group I a high incidence of bacterial endocarditis was recorded in rabbits infected with the highly serum resistant strain SM 55 and with the moderately serum resistant strain SM 104, while none of the rabbits challenged with the serum sensitive strain CDC O13 developed infection ($p<0.001$) (Table 1) In group II the incidence of bacterial endocarditis was 3 out of 5 due to both SM

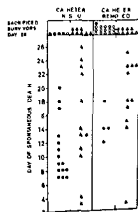


Fig 3 Survival time of rabbits with *Serratia marcescens* endocarditis Each dot represents one rabbit inoculated with SM 104 (● infected vegetations O non infected vegetations) or with SM 55 (▲ infected vegetations △ non infected vegetations)

55 and SM 104 one day after challenge How

104 than due to the more resistant strain SM The different incidence of endocarditis due to 104 in groups I and II is highly signifi ($p<0.001$), whereas the incidence of SM infections was not significantly different in the groups

Survival Time Related to Presence or Absenc Catheter

Fig 3 shows the survival times of rabbits died spontaneously of endocarditis and the nur of surviving rabbits sacrificed 28 days challenge The number of early deaths (*i e* surv time <12 days) is significantly higher in ral with an indwelling catheter (14/27) than in t lacking a catheter (1/15) ($p<0.001$) Most of rabbits with an indwelling catheter which surv for 28 days still had infected vegetations w most of those without a retained catheter had st vegetations or macroscopically normal valve l lets This is particularly true for rabbits challer with the moderately serum resistant strain SM 1

Appearance Weight and Bacteriology of Vegetat

Distribution of vegetations was different rabbits with and without an indwelling cath during the experimental period Rabbits withou catheter had their vegetations chiefly on the ao valve leaflets, while rabbits with an indwell catheter also had large vegetations outside the ao valve leaflets, *i e* on the wall of the aorta and al the catheter on the wall of the left ventricle It w regular feature that only one or two of the th aortic valve leaflets was affected and that vegetations remained limited to the origin

ur results are in agreement with observations e in human infections which show that eraemic *S marcescens* strains are invariably m resistant (15) although »delayed serum ntive« strains isolated from the blood stream been described recently (16) The influence of catheters was emphasized by *Wilfert et* 17) who found that in 44% of 39 episodes of an *S marcescens* bacteraemia the indwelling ter was the probable portal of entry

he presence of an indwelling catheter in the rimental model led to continuous enlargement he vegetations not only at the aortic valve ts but also along the catheter below and above valves (8) The histological appearance of the e vegetations in the rabbits pretreated for carditis however is different on the aortic leaflets and outside these as earlier described indicating that »bacterial aggregation and th in these two types of vegetations might be ent« Later investigations of the spontaneous e of *S faecalis* (9 10) and the present tigation of *S marcescens* endocarditis seem to f these early observations The histological inations showed that the density of bacteria very high in all vegetations on the aortic valve ts but only small foci of bacteria were found de the valves Quantitative cultures showed ten veral hundred times difference in bacterial ty in these two types of vegetations (unpub l work) Similar observations were published tly by *Francioli & Freedman* (6) who compared acterial density of *Streptococcus sanguis* in the ations of aortic and tricuspid valves with in different parts of the vascular system de the heart The lower mean number of CFU/

in this way vegetations from different sites with different bacterial density will influence ean value

e role of the catheter during pretreatment (8 9) ely mechanical The later adhesion of bacteria vegetations and the development of infection ake place without the presence of the catheter acteria act as small foreign bodies and their h will lead to continued deposition of fibrin thrombocytes thus increasing the size of tions The actual extension of vegetations may e result of a subtle balance between forces ring a deposition of fibrin and thrombocytes other forces provoking a diminution of the ations such as mechanical separation of small s organization of the vegetations by development of small new vessels and proliferating fibroblasts and probably also fibrinolytic action on the vegetations by serum factors as described earlier (8) The extent of these latter processes is underlined by the surprisingly rapid disappearance (4–10 days) of the sterile vegetations developed after 3 days with catheter *in situ*

The role of the indwelling catheter during the infectious process is not limited to its mechanical effect It probably influences the physiological processes in its proximity thereby upsetting the balance between the forces involved and resulting in delayed recovery Our results demonstrate that an indwelling catheter will affect both the frequency of infection establishment and the outcome of the endocarditis The higher incidence of endocarditis 1 day after challenge the higher weight of vegetations and the higher number of CFU/g vegetation in rabbits with an indwelling catheter compared with those without a catheter may be due to a more rapid covering of both bacteria and catheter with fibrin which affords protection against humoral and cellular defence mechanisms These physiological changes induced by the catheter are probably also responsible for the significantly higher incidence of early death in rabbits with an indwelling catheter Thus the course of infection will be acute and severe

Altogether the results show that an indwelling catheter contributes to the maintenance of an infection which would take a significantly different course without the catheter depending on the degree of serum resistance of the infecting strains Therefore it is our opinion that an experimental model using an indwelling catheter is not ideal and should not be used indiscriminately

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ml blood occurred intermittently, such values were without predictive importance for the time of spontaneous death The level of bacteraemia in rabbits which died spontaneously of endocarditis was slightly elevated terminally, but there were no signs of an overwhelming generalized infection

Spleen

In 33 rabbits without evidence of endocarditis (28 days observation), the mean weight of the spleens was 1 18 g (SD 35%), *i e* significantly lower than in 57 rabbits with infected vegetations (2 39 g, SD 83%) No significant differences occurred between the spleen weights of rabbits inoculated with SM 55 (mean 2 36 g) and SM 104 (mean 2 41 g) A spleen weight >2 g was found in 34/57 rabbits (59 6%) with infected vegetations, but only in 1/33 rabbits without signs of endocarditis Splenic infarction was recognized in 3/130 rabbits

Kidneys

In rabbits with an indwelling catheter and evidence of bacterial endocarditis, 69 out of 78 kidneys (88%) had one or more infarcts, while in rabbits lacking an indwelling catheter, 24 out of 40 kidneys (60%) had infarcts There was no significant difference between groups of rabbits infected with SM 55 and with SM 104 The incidence of large infarcts (≧10 mm^2) was slightly higher in rabbits infected with SM 104 (34/41 infarcted kidneys) than in those infected with SM 55 (33/51)

Other Organs

The weights of the hearts were not significantly different in rabbits with infected vegetations due to SM 55 and SM 104 (28 days observation) The mean weight of hearts in all rabbits with evidence of endocarditis was 9 23 g (SD 24% 52 observations), while in all rabbits without evidence of endocarditis it was 8 30 g (SD 24% 32 observations)

Focal infectious processes (except for the kidneys) yielding growth of *S marcescens* were seen as subphrenic abscesses or abscesses in the liver in 4/130 rabbits Three rabbits had neurological symptoms with paralysis of their legs Pericardial effusion of 2–5 ml was found in 50/130 rabbits while pleural and peritoneal effusions more than a few ml were seldom recorded

Rabbits with heavily infected vegetations (≧10^9 CFU/g) nearly always had positive blood cultures terminally and also heavy growth of *S marcescens* from the spleen, liver, kidneys and, to a smaller extent, from the lungs and mesenteric glands On the other hand, rabbits with slightly infected vegetations (<10^9 CFU/g) had negative blood cultures terminally and no growth from the organs

DISCUSSION

The three *S marcescens* strains used to pr[illegible] endocarditis varied in two characters degr[illegible] serum susceptibility and proteolytic capacity H[illegible] ver, pathological and clinical data indicati[illegible] separate effect due to the proteolytic capacity [illegible] strains were not obtained This was [illegible] since it had earlier been shown by us (10) proteolytic strains of *Streptococcus faecalis* pr[illegible] ced a more severe and acute course of endoca[illegible] characterized by a shorter survival time a[illegible] higher degree of bacteraemia with small amour[illegible] soft, friable vegetations in the left side of the [illegible] and a large number of kidney infarcts than [illegible] proteolytic strains, probably as a result of p[illegible] dissolution of fibrin in the vegetations Why very strong *in vitro* proteolytic activity of th[illegible] *marcescens* strain SM 104 was not apparent [illegible] *vivo* conditions is not clear, theoretically it migh[illegible] due to a blocked production of proteases insid[illegible] vegetations or inhibition of native protease in[illegible] tors of rabbit plasma The latter possibility [illegible] support in the work of *Hochstrasser et al* where it is shown that the proteases of *Pseudom[illegible] aeruginosa* strains are practically completely in[illegible] ted by human and animal α_2-macroglobulin

The different rates of infection and spontan[illegible] death observed in our experimental model o[illegible] *marcescens* endocarditis appear to be due to [illegible] effect of the serum bactericidal system In rab[illegible] with an indwelling catheter none of the anim[illegible] could be infected with the serum sensitive str[illegible] whereas nearly all rabbits were infected with [illegible] moderately and the highly serum resistant stra[illegible] This is in accordance with *Archer & Fekety's* observation that serum sensitive strains of *Pseu[illegible] monas aeruginosa* failed to infect sterile vegetati[illegible] in their rabbit model of endocarditis using [illegible] indwelling catheter during the experimental peri[illegible] Similarly, using seven serum resistant and sev[illegible] serum sensitive strains of *Escherichia coli* *Dura[illegible] & Beeson* (4) showed that the ability of the strains [illegible] establish endocardial infections was directly associ[illegible] ted with the resistance to the complement mediat[illegible] serum bactericidal system Experiments with t[illegible] serum resistant *S marcescens* strains showed th[illegible] rabbits infected with a highly resistant strain had significantly higher incidence of endocarditis (75%[illegible] than those infected with the moderately seru[illegible] resistant strain (25%) Such a difference could onl[illegible] be demonstrated in rabbits with no indwellin[illegible] catheter during the experimental period and ther[illegible] fore an indwelling catheter must be assumed to b[illegible] able to camouflage minor differences in serum susceptibility

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THE GLUTARALDEHYDE-FIXED CYTOCENTRIFUGE-PREPARED URINE SEDIMENT IN THE EVALUATION OF URINARY TRACT INFECTION

The Significance of Pyuria

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rberg B Norberg A & Gippert H *The glutaraldehyde fixed cytocentrifuge prepared urine* ment in the evaluation of urinary tract infection The significance of pyuria Acta path microbiol nd Sect B 88 277–279 1980

ring antibiotic treated urinary tract infection in non-catheterized geriatric inpatients bacteriuria appeared within 1–2 days Pyuria on the other hand disappeared within 4–7 days After hdrawal of antibiotic therapy bacteriuria reappeared quicker than pyuria It is suggested that the e lag between bacteriuria and pyuria is due to the fact that bacteriuria reflects bacterial invasion into urinary tract whereas pyuria reflects the inflammatory response of the urinary tract

y Words Urinary tract infection bacteriuria pyuria urine sediment

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d 24 iii 80 Accepted 9 vi 80

conventional urine sediment is distinguished or reliability and poor validity The back l of this nuisance is complex The main fac hich invalidate the urine sediment are varying content in the urine bacterial growth after ng cell and cast disintegration after sam varying volume of resuspension urine g volume of resuspended urine droplet on the poor contrast of specimens and poor micro resolution (cf 1–9)

have tried to offset the weaknesses of the tional urine sediment by the development of ment quantified (PQ) urine preparation This ent was distinguished by standardized sam immediate fixation with glutaraldehyde cyto fuge preparation and contrasting with routine (5 6 7)

the evaluation of urinary tract infection (UTI) a has been a matter of dispute (1 3 4 9) The nt study was de[illegible] elucidate the course and role of pyuria in bacterial UTIs of non catheterized geriatric inpatients by means of the PQ urine sediment

MATERIAL AND METHODS

This study was conducted on 30 consecutive non catheterized in patients from the psychogeriatric wards of Saint Lars Hospital in Lund The patients were admitted to the Department of Internal Medicine because of clinical symptoms of UTI bacteria and/or leukocytes in a conventional urine sediment and a positive routine quantitative urine culture Prior to the urine sampling the orificium of the urethra was cleared with sterile saline The control samples prior to antibiotic treatment were delivered as two non morning midstream urine samples with an interval of two hours The urine was collected in a sterile plastic tube and thoroughly shaken Then 0 1 ml urine was pipetted to a glass tube with 0 9 ml 2% glutaraldehyde in 0 134 M phosphate buffer pH 7 4 The patients were allocated at random to double-blind treatment with pivmecillinam HCl 200 mg 3 times

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and pyuria reflect entirely different gh correlated phenomena The bacteriuria cts the bacterial invasion into the urinary tract pyuria reflects the inflammatory response of urinary tract

he present observations on the relation between eriuria and pyuria are in agreement with the cal model of a primary bacterial infection and a ndary inflammatory response of engaged tissue significance of bacteriuria and pyuria has no t been recognized by many clinicians The rpretation of various combinations and magni s of bacteriuria and pyuria has nevertheless subject to keen interest from previous workers 1 3 4 9) The contribution of the present study at the time course of bacteriuria and pyuria in treated UTI has been mapped by means of rmanent quantitative urine sediment

he clinical implications of the present study are

Bacteriuria without pyuria should not be ted in the absence of imperative clinical symp s the patient seems not to be hurt by this terial invasion since there is no inflammatory ponse

Treatment of a UTI may lead to a sterile ria, which will disappear spontaneously

thank Mrs *Patricia Wetterberg* for revising the ish This study was supported by grant No 5362 the *Swedish Medical Research Council* to *Astrid berg*

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daily, or the combination pivmecillinam HCl 100 mg and pivampicillin 125 mg 3 times daily, for 7 days Urine was sampled for the PQ sediment on days 0–7, 9, 24 The glutaraldehyde-fixed urine was kept under refrigeration for 1–7 days before the preparation of the slide specimens The slide preparations were obtained by means of a cytocentrifuge (Shandon Elliot Cytospin[R]), 1000 r p m, 10 minutes, 0 2 ml glutaraldehyde-suspended urine, double preparations from each sample One of the twin preparations was stained according to May-Grunwald Giemsa, the other by means of hematoxylin-eosin The cells and bacteria were counted in a Zeiss Photomicroscope at ×1,000 magnification along a diameter of the preparation (52 visual fields) and converted into corpuscles per µl urine in the original sample, as described in a previous study (5) The twin preparation with the highest cell count was taken to represent the cellularity of the urine

RESULTS

There was a time lag between the bacteriuria and the pyuria in both the mecillinam-treated group of patients (Fig 1) and the combination treated group of patients (Fig 2) The bacteriuria disappeared completely within 1–2 days of antimicrobial therapy The pyuria was reduced more slowly an approached insignificant levels, 10 cells/µl urine, o day 4 (Fig 2) and day 7 (Fig 1), respectively Bacteriuria reappeared quicker than pyuria afte withdrawal of antimicrobials (Figs 1, 2)

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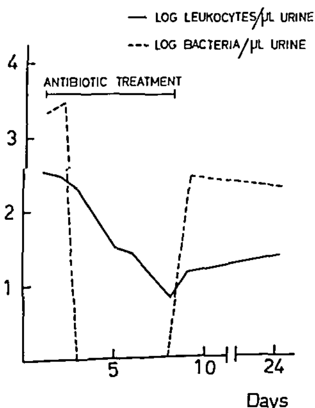


Fig 1 The time lag between bacteriuria and pyuria during mecillinam treated urinary tract infection in 15 geriatric inpatients without indwelling catheters Median values 1 = 10 cells/µl urine

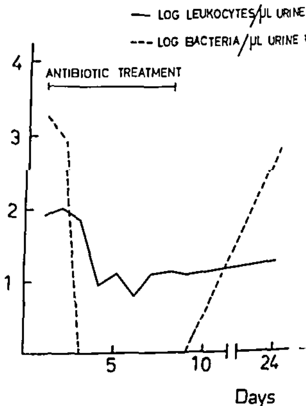


Fig 2 The time lag between bacteriuria and pyuria during urinary tract infection treated with the combination mecillinam ampicillin in 15 geriatric inpatients without indwelling catheters Median values 1 = 10 cell/µl urine

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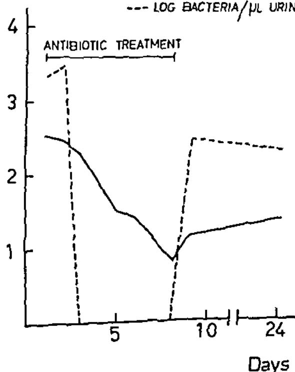


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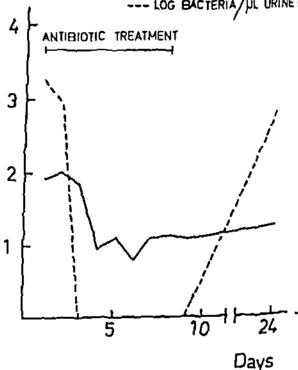


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A PROTEIN ANTIGEN CHARACTERISTIC OF *BRANHAMELLA CATARRHALIS*

Serological Identification of the Genus

INGVAR ELIASSON

Department of Medical Microbiology University of Lund Sweden

iasson I A protein antigen characteristic of *Branhamella catarrhalis* Serological identification of the nus Acta path microbiol scand Sect B 88 281–286 1980

ecip tation patterns of sonicated acid-extracted and other extracts from Branhamella catarrhalis were amined by double diffusion in gel technique using antiserum to B catarrhalis Acid extract gave rise 4 distinct precipitates One of these lines was further studied The bacterial component responsible r this line was trypsin-sensitive indicating that it was a protein It was anodally localized by crossed nmunoelectrophoresis By absorption of antiserum with whole bacteria the precipitating capacity of e serum was diminished suggesting that the protein antigen (P antigen) was exposed on the bacterial rface F(ab)₂-fragments of IgG from antiserum but not from normal rabbit serum precipitated the antigen indicating that it was a true antigen antibody reaction It was possible to make an IgG eparation monospecific for the P antigen by absorbing antiserum with trypsinized bacterial extract 1 strains of *B catarrhalis* 9 strains of *N gonorrhoeae* 10 strains of *N meningitidis* 12 other *eisseria* spp and 2 strains of *H influenzae* were investigated for presence of cross reacting surface ntigens using IgG monospecific for the P antigen and ^{125}I labelled protein A from *Staphylococcus ureus* After antibody exposure all 31 strains of *B catarrhalis* showed abundant uptake of protein A o significant uptake was detected on any other investigated strain Hence the P antigen appears to be haracteristic of *B catarrhalis* The possibility of a serological identification of the species is ntroduced Precipitating antibodies against the P antigen were demonstrated in 69% of normal human era

Key words *Branhamella catarrhalis* antigens serological identification protein A

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1970 the former name *Neisseria catarrhalis* transferred to a new genus *Branhamella* (3) new generic name was proposed on the basis of parison of DNA base content between *B rrhalis* the type species of genus *Neisseria* and 6 r *Neisseria* species

udies on cell envelope proteins (17) and ular and free lipopolysaccharides (7) also indicate there are extensive differences of cellular position and physiology between the genus *sseria* and the new genus *Branhamella*

Moreover reports published during the last ... her commonly held view of *B catarrhalis* as merely being a harmless saprophyte of the upper respiratory tract A pathogenic role of this bacterium has been indicated in acute otitis media (4 8) maxillary sinuitis (2) chronic bronchitis and bronchopneumonia in the compromised host (14) and acute laryngitis in the adult (18) Case reports concerning bacteremia with meningitis have also been published (5)

The present report concerns a protein antigen from *B catarrhalis* characteristic of this species This antigen could be precipitated by a majority of sera from apparently healthy human individuals by the double diffusion in gel technique

MATERIALS AND METHODS

Bacterial Strains

Twentynine *B catarrhalis* strains isolated from clinical specimens were identified as described by *Berger* (1) *N spp N flavescens* ATCC 13120 *N subflava* ATCC 11076 *N flava* ATCC 14221 *N perflava* ATCC 10555 *N sicca* ATCC 9913 *N canis* 14687 *N caviae* ATCC 14659 *N cinerea* 194 (*U Berger* 1973) *N ovis* ATCC 19575 *N mucosa* ATCC 19598 *N hemolysans* ATCC 10379 *N gonorrhoeae* ATCC 11688 11689 SS 11413/40 19827/40 *N lactamicus* ATCC 23970 and *Branhamella catarrhalis* ATCC 8193 NCTC 4103 were kindly supplied by Dr *I Lind* Statens Seruminstitut Copenhagen Denmark and *N* spp *N meningitidis* types A B C D X Y Z W 135 29E *N gonorrhoeae 670 wd 670 gr P9 wd P9gr KHJ F6 (W/III) N lactamicus N sicca N subflava* by Dr *D Danielsson* Regionsjukhuset Örebro Sweden Two *H influenzae* strains one type b with capsule and one not typed without capsule were isolated from clinical specimens in our laboratory

The strain listed as *N sicca* ATCC 9913 did not correspond to the fermentative criteria described for *N sicca* This strain had smooth colonies and could not utilize sugars The strain should probably be considered as a strain of *N caviae* This observation has been made independently by others (16)

Conditions for Cultivation

For antibody binding experiments (see later) *N meningitidis* and *N gonorrhoeae* were cultured on GAB medium agar plates (13) at 37 °C in an 8% CO_2 atmosphere The bacteria were harvested using sterile cotton swabs washed and suspended in PBS (phosphate buffered saline 0 03 M phosphate 0 12 M NaCl pH 7 2 + 0 02% Na azid) The other *Neisseria* spp and the *B catarrhalis* strains were cultured in 5 ml Todd Hewitt broth for 18 h at 37 °C under agitation washed and suspended in PBS *H influenzae* strains were cultured on hematin agar plates as above

Extraction Procedures

For extraction by sonication 300 ml of Todd Hewitt broth was inoculated with a 5 ml overnight culture of the strain to be investigated and incubated for 18 h at 37 °C on a rotating platform shaker operating at 120 rev/min The *N gonorrhoeae* strains were cultivated on 2 GAB medium agar plates each and the *H influenzae* strains on each 2 hematin agar plates these strains were harvested as described above Cells were recovered by centrifugation for 20 min at 3000 *g* washed twice and suspended in 5 ml of PBS Sonication lasted 15 min at 4 °C in a Sonifer *B 12 sonicator* (Branson Sonic Power Co Conn USA) The cell free supernatant obtained after centrifugation for 30 min at 3000 *g* was kept at −20 °C until used Hot acid extraction (as used by Lancefield for extraction of streptococcal antigens (9)) was prepared from 10 one litre Todd Hewitt broths in baffled flasks in which the bacteria were cultured for 18 h at 37 °C on a platform shaker operating at 120 rev/min Cells were recovered by continuous centrifugation at 5000 *g* washed three times in saline (0 9% w/v NaCl aq dest) and suspended in 100 ml of saline The pH was adjusted to 2 0 with 1 M HCl The suspension was heated for 10 min in a boiling water bath cooled on and pH readjusted to 7 0 with 1 M NaOH The cell free suspension obtained after centrifugation for 30 min 3000 *g* was stored at −20 °C until used as acid extract Hot neutral (pH 7 0) and hot alkaline (pH 10 0) extracts of *B catarrhalis* strain 235 L were prepared procedures similar to the hot acid extraction except the different pH when boiling In some experiments the acid extract or an intact bacterial suspension was digested with trypsin (SIGMA Chemical Co USA) 1% (w/ for 1 h at 37 °C The reaction was stopped by addi soya bean trypsin inhibitor (SIGMA) 1% (w/v)

Sera and Immunoglobulin Preparations

Sera were raised against *B catarrhalis* strain 235 L three rabbits and against strain 236 E in one rabbit usi the method described by *Rotta et al* for preparation streptococcal antisera (15) Porcine anti rabbit seru IgG (heavy and light chains) was purchased from DAK immunoglobulins Copenhagen Denmark Pooled h man IgG batch no 70972 was purchased from KAB Stockholm Sweden Normal human sera were collect from 90 healthy blood donors

For preparation of F(ab)₂ fragments of rabbit seru IgG 5 ml of rabbit antiserum to *B catarrhalis* strain 23 L was separated on a Sephadex G 200 column (2 5 × 9 cm) equilibrated with PBS (flow rate 18 ml/h fractio 4 5 ml as for the following columns) Each fraction w tested by immunodiffusion for presence of IgG T fractions containing IgG were pooled concentrated to ml using a DIAFLO pm 10 ultrafiltration membran (AMICON Corp USA) and dialysed against 0 07 M sodium phosphate buffer pH 6 3 The sample was the passed on a DEAE column (2 2 × 17 cm Pharmaci Fine Chemicals AB Uppsala Sweden) using the sam buffer The excluded fractions containing IgG we pooled concentrated to 5 ml and dialysed against 0 1 M sodium acetate buffer pH 4 5 After determination of th protein content (11) the sample was digested with pepsi (SIGMA Chemical Co USA) at a pepsin/IgG ratio o 1 50 (w/v) for 15 h at 37 °C and passed on an AcA 2 column (2 2 × 17 cm LKB Stockholm Sweden) usin 0 1 M sodium acetate buffer pH 4 5 The fractions of th first peak of the elution curve were pooled an concentrated to 5 ml Part of the preparation (0 2 ml) was absorbed with *S aureus* Cowan I (NCTC no 8530 in order to remove any remaining Fc fragments and th absorbed preparation was tested in immunodiffusion fo presence of F(ab)₂ fragments

Fab fragments of polyclonal human IgG were kindly supplied by Dr *A Grubb* Malmo

For absorption of serum with whole bacteria 1 ml of serum was mixed with 0 25 g (wet weight) of bacteria and incubated for 30 min at 37 °C Cells were removed by centrifugation and the supernatant was used as absorbed antiserum

To prepare rabbit IgG specific for the P antigen 5 ml of pooled antisera (from two rabbits) raised against *B catarrhalis* 235 L was mixed with 10 ml of trypsinized

tract, prepared from the same strain and ed for 30 min at 37 °C under mild agitation The a.e was removed by centrifugation for 30 min at The supernatant was concentrated to 5 ml using FLO ultrafiltration membrane pm 10 and ed on a Sephadex G 200 column equilibrated BS The fractions containing monomer IgG as from the elution volume and tests with anti rabbit rum were pooled and used as absorbed IgG (pool volume 45 ml i e diluted 1 10 in relation orig nal volume)

ochem cal Methods

ble diffusion in gel was performed in 0 6 % (w/ ose (Sea Kem /ME/ Marine Colloids Div FMC USA) in PBS The plates were read after 1 and 2 at +4 °C and stained with Coomassie Brilliant

ssed immunoelectrophoresis (10) was carried out

ds the anode of plate of the 1st dimension a 3 × 45 strip was cut and the strip placed edge to edge with tiserum containing gel (1 2 or 4 % v/v of antiserum 1 mm thick The antigens separated in 1st sion were forced into the antibody containing gel

'ion of Surface Antigen by the Use of Absorbed erum and ^{125}I labelled Protein A

Protein A was purchased from Pharmacia Uppsala den, and labelled with ^{125}I as described by onahey & Dixon (12)

he bacterial strains to be investigated were heat d (56 °C 30 m n) washed 3 times and suspended in to an absorbance of approximately 0 80 at 540 nm bacterial suspension 0 5 ml was mixed with 100 µl bsorbed IgG solution (see above) diluted 1 4 and bated for 30 min at 37 °C Two ml of PBS aining 0 1 % (v/v) Tween 20 (KEBO AB Stockholm eden) was added the suspension submitted to trifugation for 15 min at 3000 g the supernatant oved and the cells re-suspended in 0 5 ml of PBS 7 µl of ^{125}I labelled protein A (1 µg) was added lowed by incubation for 30 min at 37 °C After shing in 2 ml of PBS Tween and centrifugation the ioactivity in the bacterial pellets was counted The ioactivity found was expressed as a percentage of the al amount added

RESULTS

Precipitation Patterns Obtained in Immunodiffusion Using Extracts from B catarrhalis *and Rabbit Antiserum to* B catarrhalis

Sonicated extract of *B catarrhalis* strain 235 L was tested in double diffusion in gel for presence of precipitating components using antiserum to *B catarrhalis*

Four distinct precipitation lines were obtained among which the one appearing closest to the extract application well was more heavily stained than the others This line will be referred to in the following as the P line and the component respon sible for the precipitate as the P-component

Acid neutral and alkaline extracts of strain 235 L were also tested for content of precipitating components At least three of the components including the P-component were readily extractable by all three methods as judged from experiments in which interference with complete fusion was obta ned when the extracts

F(ab')$_2$-fragments of rabbit immune serum (raised against strain 235 L) – but not of normal rabbit serum – precipitated the P-component indicating that the P line was formed as an antigen antibody interaction

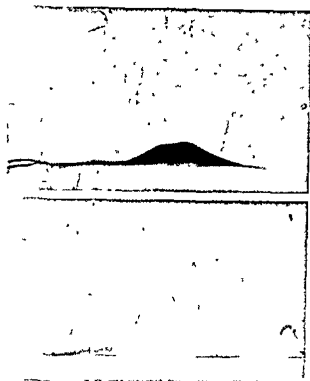
Detection of Antibodies in Human Sera against the P Component

Commercial human IgG and a total of 90 blood donor sera were examined by immunodiffusion for presence of antibodies to acid extract of strain 235 L. Commercial human IgG gave rise to two lines one of them fusing completely with the P line obtained with rabbit immune serum to *B catarrha lis* This line disappeared when equal volumes of human polyclonal Fab fragments (2 5 mg/ml) and *B catarrhalis* extract were mixed prior to testing against the commercial human IgG In all 69 % of normal human sera precipitated the P-component A few human sera also showed a second line which was not further studied

Characterization of the P Component

The P line disappeared after trypsin digestion of hot acid extract while the other antigens were unaffected when tested by immunodiffusion in-gel

Trypsin-digested and crude hot acid extracts were examined by crossed immunoelectrophoresis Using this method it was possible to distinguish five separate precipitation lines One line appearing only in untreated extract, was apparently the P line This



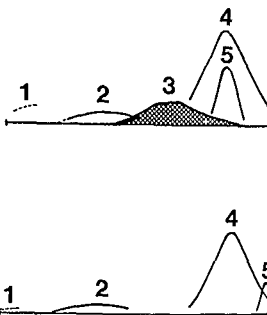


Fig 1 Crossed immunoelectrophoresis of extracts from *B catarrhalis* strain 235 L 4% of rabbit antiserum *B catarrhalis* was incorporated in the gel Anode to the right
Top Untreated acid extract No 3 represents the P antigen Bottom Trypsinized extract Note that precipi No 3 is absent and that the position of line No 5 is altered towards the anode

line was anodally localized in the crossed immunoelectrophoresis One other precipitating component was altered by the trypsin digestion, moving more anodally after treatment of the extract The other three precipitating components of the extract kept the same electrophoretical mobility (Fig 1)

Rabbit antiserum to *B catarrhalis* was absorbed with *B catarrhalis* strain 235 L The absorbed antiserum was unable to precipitate with the P-antigen or any of the other components precipitated by unabsorbed serum Moreover, absorption of the serum with trypsin digested bacteria abolished all lines formed between the extract and unabsorbed serum

After separation of the acid extract on a Sephadex G-200 column, equilibrated with PBS the P-antigen was found in the void volume

Investigation of B catarrhalis, Neisseria *spp and* H influenzae, *Using Absorbed IgG Directed against the P-Antigen and* ^{125}*I-Labelled Protein A*

The extract-absorbed rabbit IgG was tested by immunodiffusion for the presence of antibodies against acid extract of *B catarrhalis* As judged by this method, it was monospecific to the P-antigen

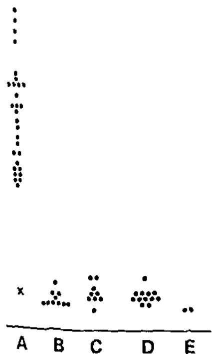
0 5 ml volumes of the standard suspension (see above) strain 235 L were mixed with 100 µl absorbed IgG in various dilutions Subsequ addition of protein A gave the following uptakes *B catarrhalis* undiluted IgG solution, 91% uptal IgG solution diluted 1 2, 90%, 1 4, 59%, 1 33%, 1 16 17% IgG solution diluted 1 4 w used in the following experiments

To examine the effect of the quantity of bacteri on the protein-A uptake the bacterial suspensio used above was diluted in twofold steps and 0 5 n of each dilution was mixed with 100 µl of absorbe IgG solution diluted 1 4 The results were a follows undiluted suspension 58%, suspensio diluted 1 2, 52%, 1 4 46% 1 8 37%, 1 1 28% 1 32 19% 1 64 13% and suspensio diluted 1 128 10% uptake A standard suspensio of strain 235 L mixed with rabbit preimmun serum gave an uptake of less than 4%

The protein A uptake on antibody-expose

longing to different types) and 12 different othe *Neisseria* spp (one strain of each) Two strains of *H influenzae* were also included (one encapsulate,

establish the presence of P antigen in all strains No other strain of other species used in the study contained P antigen as judged by this method



Binding of ^{125}I labelled protein A to different after addition of absorbed IgG antibodies to the m.

result with preimmune serum and *B catarrhalis* 35 L Each mark represents the mean value of alts obtained with one strain

B catarrhalis strains B – *N meningitidis* C *N gonorrhoeae* strains D – apathogenic species and E – *H influenzae* strains

b and one not typed without capsule) The are shown in Fig 2

B catarrhalis strains all showed an uptake in of 13% (mean 20 5% SD 5 7%) while the strains examined all gave less than 5% (mean SD 0 7%) binding of protein A The latter was not higher than that obtained with *B catarrhalis* and preimmune serum (mean 3 5%)

extracted extract of 10 strains of *B catarrhalis* examined for presence of P antigen by double sion in-gel technique using acid extract of 235 L as a reference and rabbit antiserum to *catarrhalis* By this method it was possible to

DISCUSSION

Extracts of *B catarrhalis* were found to give several precipitation lines in immunodiffusion against rabbit antiserum to *B catarrhalis* one of the lines referred to as the P line was more prominent than the others The formation of the P line between hot hydrochloric acid extract and F(ab')$_2$-fragments of IgG from rabbit immune serum (but not with such fragments of normal rabbit serum) indicated that the P line resulted from an antigen antibody reaction Moreover the formation of the P line between polyclonal human IgG and the extract was inhibited by addition of Fab-fragments of polyclonal human IgG to the extract These findings indicated that the P line was probably not the result of a reaction between *B catarrhalis* component and antibody independent of the specificity of the antibody such a reaction has been described between IgD and *B catarrhalis* (6)

IgD myeloma protein did not precipitate with extracts of *B catarrhalis* nor did it inhibit the formation of the P line when mixed with extract before immunodiffusion (unpublished observation)

The trypsin sensitivity of the bacterial component (P antigen) responsible for the P line indicates that it is a protein

By absorption of rabbit antiserum to *B catarrhalis* with trypsinized extract of the same strain it was possible to prepare an antiserum specific for the P antigen When using this absorbed serum and radio-labelled protein A from *Staphylococcus aureus* the P antigen could be detected as a surface antigen on the intact bacteria Significant antibody binding to *B catarrhalis* was detected in amounts of bacteria down to 1% of the quantity of bacteria used in the studies designed to detect P antigen among different bacterial species In all 31 strains of *B catarrhalis* 31 of *Neisseria* species and 2 *H influenzae* strains were investigated regarding their capacity to bind anti P antibodies The results revealed an abundant uptake in all 31 strains of *B catarrhalis* but no significant uptake in other species indicating that the P antigen is a surface structure characteristic of *B catarrhalis*

The variable results 13–38% binding of the different *B catarrhalis* strains were not further studied in this investigation Among other explanations this might simply reflect differences in the concentrations of the standard suspensions

In studies concerning the taxon relatedness of *B*

catarrhalis, the genus *Neisseria* and other genera, more work is needed to elucidate the antigenic relationship Studies on extracellular antigens have not revealed any cross-reactions between *B catarrhalis* and *Neisseria* species (16) To my knowledge, no protein antigen with characteristics similar to the P-antigen has yet been identified

In the present investigation, a total number of 90 human sera were tested by immunodiffusion for the ability to precipitate hot acid extracts of *B catarrhalis* 69% of the sera formed precipitates which fused completely with the line formed between the P-antigen and rabbit antiserum to *B catarrhalis* Precipitating antibodies against not further characterized structures in *B catarrhalis* have been shown by others to be common in both normal human sera and sera from individuals recently infected with *B catarrhalis* (2) A pathogenic role of *B catarrhalis* has been indicated in several studies (see above) Studies are in progress concerning the role of the P-antigen in the virulence of the bacteria, and the antibody response during infection

Identification of *B catarrhalis* has hitherto relied on biochemical tests colony morphology, and growth characteristics *B catarrhalis* is relatively inactive biochemically, and the oxidase and catalase production as well as nitrate reduction are characteristics shared with several other closely related microorganisms The fermentation tests for recognition of the bacteria are generally based on negative reactions A serological identification of this genus – by means of identification of the P-antigen – would seem to be desirable A quick and simple method for the serological identification of *B catarrhalis* on the basis of the P-antigen is under development

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STUDIES ON THE PATHOGENICITY OF *YERSINIA ENTEROCOLITICA* AND *Y ENTEROCOLITICA*-LIKE BACTERIA

Enterotoxin Production at 22 °C and 37 °C by Environmental and Human Isolates from Scandinavia

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perud G Studies on the pathogenicity of *Yersin a enterocolitica* and *Y enterocolitica* like bacteria :nterotoxin production at 22 °C and 37 °C by environmental and human isolates from Scandinavia path microbiol scand Sect. B 88 287–291 1980

gether 412 strains of *Yersinia enterocolitica* and *Y enterocol tica* like bacteria from environmental human sources were examined for their ability to produce enterotoxin at 22 °C and 37 °C using infant mouse assay A total of 73 strains were positive at 22 °C only Another 28 strains were tive both at 22 °C and 37 °C Of these 28 strains 26 were sucrose and aesculin non fermenting all were Voges Proskauer negative All of these strains were isolated from non human sources All 13 strains belonging to O serogroup 28 produced enterotoxin at 37 °C The results indicate that rotoxin production at 22 °C is widespread in *Y enterocolitica* with the highest prevalence among nan clinical isolates Enterotoxin production at both 22 °C and 37 °C seems to be common in *Y ktensen i* (sucrose non fermentors) Enterotoxin production is apparently rare in *Y frederiksenii* mnose fermentors) and in *Y intermed a* (rhamnose and melibiose fermentors)

y words *Yers nia enterocol tica* enterotoxin

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e strains of *Yersinia enterocolitica* produce a able enterotoxin *in vitro* which is active in the mouse and rabbit ileal loop assay (2 5 11 3) A close similarity between the *Y col t ca* enterotoxin and the heat stable (ST) toxin of *Escherichia coli* has been shown (2 Rob ns Browne *et al* (13) stated that, in n to sharing a number of physiochemical ties these two enterotoxins are similar in mechanism of action *Pai et al* (12) stud ed n and non human [illegible]lates from Canada and sed that ent[illegible] [illegible]duction (at room [illegible] [illegible]ocolitica with the highest prevalence among strains associated with human infection However none of the authors referred to above report enterotoxin production at the human body temperature Likewise documentation of enterotoxin production *in vivo* and under anaerobic conditions is lacking Thus a clinical significance of the *Y enterocolitica* enterotoxin has not been established

In a current communication from this laboratory it was reported that some strains of *Y enterocolitica* O-serogroup 28 produced enterotoxin at 37 °C as well as at 22 °C (9) The purpose of the present study was to survey the prevalence of enterotoxin production at 37 °C and 22 °C among

Y enterocolitica and *Y enterocolitica*-like bacteria isolated from environmental and human sources in Scandinavia

MATERIALS AND METHODS

Bacterial Strains

A total of 412 strains of *Y enterocolitica* and *Y enterocolitica*-like bacteria were examined All strains were stored as stab cultures at 4 °C Three groups of strains may be discerned (Table 1)

Environmental strains A total of 331 strains from environmental sources were examined Included are 330 strains isolated from the intestinal contents of apparently healthy, wild-living mammals birds and fish and from water samples and soil in Scandinavia Most of these strains have been described in previous communications (6, 7, 8) Also included is one strain from a diseased goat (O-serogroup 2)

Human strains Included are 48 strains from human stools Twenty of these strains were isolated from healthy humans (*Kapperud, G*, manuscript) The remaining 28 strains were isolated at the National Institute of Public Health, Oslo, (courtesy of Dr *J Lassen*) from patients with gastroenteritis Twenty-two of these strains belonged to O-serogroup 3/biotype 4 (Wauters' biotype scheme)

Reference strains A total of 33 type strains representing *Y enterocolitica* O serogroup 1–34 were examined The strains were received from Institut Pasteur, Paris

Preparation of Sterile Culture Filtrates

Sterile culture filtrates were obtained from bri cultures incubated on a roller drum (60 rev/min) room temperature (approximately 22 °C) and at 37 for 48 hours (10)

Enterotoxin Assay

The culture filtrates were tested for enteroto activity using the infant mouse assay (4) as previou described (9)

RESULTS

Of 412 isolates of *Y enterocolitica* and *Y enteroc litica*-like bacteria, 101 (25%) produced *E coli* S like enterotoxin at 37 °C and/or 22 °C as indicat by the infant mouse assay Of these 101 strains, were positive at 22 °C, only The remaining strains were positive both at 22 °C and 37 (Table 1) Among the strains examined, t prevalence of enterotoxigenicity varied with t source of isolation, O-serogroup and biochemic properties of the strains

Source of Isolation

Enterotoxin production was indicated for (19%) of 331 environmental strains, 29 (60%) 48 human strains and for 8 (24%) of 33 referen strains (Table 1) The highest prevalence of enter toxigenicity was observed among the strains isolat

TABLE 1 *Relationship between Enterotoxin Production and Source of Isolation of* Y enterocolitica *and* Y enterocolitica-*Like Bacteria*

Source	No of strains	No positive at		Total (%)
		22 °C	22 °C and 37 °C	
Environmental strains				
Shrews	35	4	14	51
Small rodents	96	26	5	32
Water	57	4	6	18
Fish	129	1	1	2
Birds	7	3	–	–
Foxes	5	–	–	–
Soil	1	–	–	–
Goat	1	–	–	–
Human strains				
Gastroenteritis	28	20	–	71
Healthy	20	9	–	45
Reference strains	33	6	2	24
Total	412	73	28	25

TABLE 2 *Relationship between Enterotoxin Production and O Serogroups of* Y enterocolitica *and* Y enterocolitica *Like Bacteria*

Serogroup	Environmental strains			Human strains		
	No tested	No positive at		No tested	No positive at	
		22 °C	22 °C and 37 °C		22 °C	22 °C and 37 °C
1	9	7	1	–	–	–
3	7	1	–	22	20	–
4	35	4	–	2	1	–
5	2	–	–	3	2	–
6	41	9	–	6	2	–
7	5	1	–	1	1	–
11	8	–	4	–	–	–
12	11	3	–	–	–	–
13	5	–	–	4	1	–
16	13	4	–	2	1	–
28	12	–	12	–	–	–
NAG*	130	9	9	7	1	–
Other	53	–	–	1	–	–
Total	331	38	26	48	29	–

*Non agglutinable

rom human patients with gastroenteritis followed by the strains from healthy wild living mammals water and fish (in order of decreasing prevalence) All the 28 strains which produced enterotoxin at 37 °C were isolated from non human sources of terrestrial and aquatic ecosystems Altogether 19 (68%) of these strains were isolated from healthy wild living small mammals Shrews (Soricidae) harboured 14 (50%) of the strains which produced enterotoxin at 37 °C Forty per cent of the strains recovered from shrews showed this property

O-serogroups

Among the environmental isolates enterotoxin production was indicated for strains belonging to the different O-serogroups and for non agglutinable strains (Table 2) Positive test was obtained for 22% of O serogroup 6 and 11% of O serogroup 4 two of the most frequently encountered O-serogroups in Scandinavian ecosystems Among the reference strains enterotoxin production was indicated for O-serogroup 5 5–27 12–25 –16 14 20 22 and 28 Further enterotoxin production was indicated for 20 (91%) of 22 strains belonging to O-serogroup 3/biotype 4 isolated from human patients with gastroenteritis However none of these strains were enterotoxigenic at the human body temperature Enterotoxin production at 37 °C was indicated for environmental strains belonging to O-serogroup 1 11 28 and non agglutinable In addition two of the reference strains showed this

Biochemical Properties

Among the environmental isolates the highest prevalence of enterotoxigenicity was observed with

37 °C were sucrose and aesculin non fermenting and all were Voges Proskauer negative (at 22 °C and 37 °C) A very low prevalence of enterotoxigenicity was detected among the rhamnose fermenting strains and among the rhamnose and melibiose fermenting strains

DISCUSSION

The results indicate that enterotoxin production is widespread among *Y enterocolitica* and *Y enterocolitica* like bacteria from Scandinavia The highest prevalence of enterotoxigenicity was observed among strains associated with human gastroenteritis However none of these clinical isolates produced enterotoxin at the human body temperature

TABLE 3 *Relationship between Enterotoxin Production and Biochemical Groups of* Y enterocolitica *and* Y enterocolitica-*Like Bacteria Isolated from Environmental Sources*

Biochemical group[a]	No of strains	No positive at		T(
		22 °C	22 °C and 37 °C	(!
Y enterocolitica (YE)	136	26	2	[illegible]
Y enterocolitica-like bacteria				
Sucrose − (S)	49	9	24	6
Rhamnose + (R)	55	1	−	
Rhamnose + melibiose + (RM)	21	1	−	
Other[b]	70	1	−	

[a] Definition of biochemical groups

YE	rhamnose −,	sucrose +,	melibiose −
S	» −,	» −,	» −
R	» +,	» +,	» −
RM	» +,	» +,	» +

All groups were positive for cellobiose, sorbose and ornithine decarboxylase

\+ = fermenting,
− = non-fermenting

[b] Other combinations of the characters listed above

Further, both humans and animals were healthy carriers of enterotoxigenic strains Thus, the clinical significance of the *Y enterocolitica* enterotoxin remains uncertain

Enterotoxin production at 37 °C was indicated for a group of sucrose and aesculin non-fermenting, Voges-Proskauer negative strains most of which were isolated from the intestinal contents of apparently healthy, wild-living small mammals Similar strains have occasionally been isolated from atypical clinical entities in humans (1) With this exception, no clinical role has so far been ascribed to such strains The present data, however, suggest that the pathogenicity of these strains should be reconsidered

Pai et al (12) reported that 64 7% of 414 *Y enterocolitica* strains from Canada were positive in the infant mouse assay after incubation at room temperature This relatively high percentage compared to the present results may be due to the dominance of human clinical isolates in their work Further, *Pai et al* (12) reported that the prevalence of enterotoxin production was 50% among strains isolated from animals They did not give any information regarding the kinds of species studied or the clinical significance of these isolates However, the reported prevalence is not significantly different from the present observation on healthy, wild-living small mammals (Table 1)

Brenner et al (3) examined *Y enterocolitica* *Y enterocolitica*-like bacteria by DNA-hybrid tion They proposed four species correspondin distinct DNA-relatedness groups all of which co be biochemically defined *Y kristensenii* (suc non-fermentors), *Y frederiksenii* (rhamnose mentors), *Y intermedia* (rhamnose and melib fermentors) and *Y enterocolitica* (typical isola The present results indicate that these species h different enterotoxigenic properties (Table 3) E rotoxin production both at 37 °C and 22 °C se to be common in *Y kristensenii* Enterot production at 22 °C, only, is widespread in *enterocolitica*, with the highest prevalence am human clinical isolates Enterotoxin productio apparently rare in *Y frederiksenii* and *Y inter dia*

Among the strains studied *Y kristens* dominated in small mammals, especially shre whereas *Y frederiksenii* prevailed in fish (*Kapper G*, manuscript) Water contained both species approximately equal proportion This condit may account for the differences in the prevalenc enterotoxin production observed between stra from small mammals, fish and water (Table 1)

A clinical significance of the *Y enterocolit* enterotoxin could not be established in this wo Recently, however, it was shown that some stra of *Y enterocolitica* and *Y enterocolitica*-like bac

n produced enterotoxin at refrigeration tempera ture which is a common storage condition for foods (10) A possible role of this enterotoxin in food poisoning has been proposed (2, 10) The omnipre sence of enterotoxigenic strains of *Y enterocolitica* and *Y enterocolitica* like bacteria in nature, as recently indicated suggests that this topic deserves further attention

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STUDIES ON THE PATHOGENICITY OF *YERSINIA ENTEROCOLITICA* AND *Y. ENTEROCOLITICA*-LIKE BACTERIA

2 Interaction with HeLa Cells among Environmental and Human Isolates from Scandinavia

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Kapperud G Studies on the pathogenicity of *Yersinia enterocolitica* and *Y enterocolitica* like bacteria 2 Interaction with HeLa cells among environmental and human isolates from Scandinavia Acta path microbiol scand Sect B *88* 293–297 1980

A total of 412 strains of *Yersinia enterocolitica* and *Y enterocolitica* like bacteria were examined for their ability to interact with HeLa cell monolayers Of 331 isolates from environmental sources in Scandinavia only three strains biochemically classified as *Y pseudotuberculosis* were invasive for HeLa cells Invasiveness was also indicated for one strain (O serogroup 2) from a diseased goat Another eight strains adhered firmly to the cell surface in great numbers All of 22 strains belonging to O serogroup 3 from human patients with gastroenteritis were invasive Seven strains of O serogroup 3 from small rodents and water were non invasive Among 33 reference strains representing *Y enterocolitica* O serogroup 1–34 invasiveness was indicated for strains with known pathogenicity (O serogroup 1 2 3 5–27 8 9) However some strains belonging to O-serogroups with uncertain clinical significance were also invasive for HeLa cells A close correlation between invasiveness and enterotoxin production was demonstrated for the 22 human clinical isolates belonging to O sero group 3

Key words *Yersinia enterocolitica* invasiveness HeLa cells

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The ability of certain strains of *Yersinia enterocolitica* to penetrate HeLa cells has been reported by several authors (6 8 9 10) This property seems to be closely related to the pathogenicity of *Y enterocolitica* since almost exclusively strains with recognized clinical significance were invasive for HeLa cells *Une* (9) concluded that this model reflected well the ability to invade epithelial linings in vivo Therefore invasion of HeLa cells might serve as an indicator of the pathogenicity of *Y enterocolitica* [illegible]

In the present study, HeLa cell invasiveness was investigated in a strain material previously examined for the ability to produce enterotoxin (5) Enterotoxin production is widespread among *Y enterocolitica* and *Y enterocolitica*-like bacteria from Scandinavia The highest prevalence of enterotoxigenicity was observed among human clinical isolates However, enterotoxin production was also indicated for a broad spectrum of strains with uncertain clinical significance

The aims of the present investigation were 1) to survey the ability to penetrate HeLa cells among *Y*

enterocolitica and *Y enterocolitica* like bacteria from environmental and human sources in Scandinavia (1) to ascertain if any correlation is portrayed between HeLa cell invasiveness and enterotoxin production

MATERIALS AND METHODS

Bacterial Strains

A total of 412 strains of *Y enterocolitica* and *Y enterocolitica* like bacteria and seven reference strains of *Y pseudotuberculosis* were examined The strains have been described in a concurrent article (5) Three groups of strains may be discerned

Environmental strains 331 isolates from varied ecological and geographical sources in Scandinavia (Table 1)

Human strains 48 strains from healthy and diseased humans in Norway (Table 2)

Reference strains 33 type strains of *Y enterocolitica* and seven type strains of *Y pseudotuberculosis* (Table 3

Preparation of Bacterial Inoculum

The strains were grown in trypticase soy broth (BBL 11767) with 0 6% yeast extract (Difco 0127) The broths were incubated at 22 °C for 18–20 hours Prior to challenge of the HeLa cells the cultures were centrifuged and the bacterial pellets were re suspended in identical volumes of Eagle s basal medium (BME) with sodium bicarbonate (1g per ml) and 5% foetal bovine serum (FBS)

Cultivat on of HeLa Cells

supplied with 1 ml cell suspension in BME containing bicarbonate and 10% FBS Streptomycin (100 iu per ml) and vancomycin (100 µg per ml) were added to the medium The cell inoculum was adjusted to approximately 8×10^4 cells per well to achieve a cell layer with blank areas (leopard spots) to increase the surface available for bacterial penetration The cells were incubated overnight All incubations were performed at 37 °C in 5% CO_2 atmosphere with saturated humidity

Challenge of Cell Cultures

The cell monolayers were washed three times with temperate (37 °C) Dulbecco s complete phosphate buffered saline (PBS) (pH 7 2) to reduce the presence of streptomycin/vancomycin and re supplied with fresh BME (5% FBS) without antibiotics After incubation for one hour the cells were washed with PBS (37 °C) to minimize the concentration of antibiotics Finally the cells were incubated with 1 ml fresh BME (5% FBS without antibiotics) and inoculated with 0 025 ml of a bacterial suspension containing approximately 3×10^{12} living bacteria per ml

Following an incubation for 90 minutes the medium was decanted and the cells were washed three times with PBS (37 °C) to remove extracellular bacteria Fresh BME (5% FBS) with streptomycin/vancomycin was adde The streptomycin was used to inhibit the growth of remaining extracellular bacteria and thereby protect cells from re infection The cultures were incubated three hours

Staining and Microscopy

After incubation the medium was decanted and cells were washed three times with PBS The HeLa ce were fixed with absolute methanol for one minu coverslips were removed mounted on slides and stain with 4% Giemsa solution for 30 minutes T preparates were examined by oil immersion microsco at a magnification of 1000 ×

Interpretation of the Results

The strains were considered invasive if a majority the HeLa cells contained more than 10 intracellul bacteria The number of intracellular bacteria vari considerably between individual strains However i further quantification was attempted It was sometim difficult to distinguish intracellular (invasive) fro extracellular (adhesive) bacteria In practice invasivene was evaluated by visual comparison with strain IP 1((Institut Pasteur Paris) *Lee et al* (6) confirmed t invasiveness of this strain by electron microscop Strains showing ambiguous results were retested 3- times

RESULTS

Environmental Strains

Twelve of 331 strains of *Y enterocolitica* and *Y enterocolitica* like bacteria from environmenta sources in Scandinavia interacted with HeLa cell (Table 1)

Only four (1%) strains were invasive One c these strains (O serogroup 2) was isolated from diseased goat Invasiveness was also indicated fo three strains biochemically classified as *Y pseudotuberculosis* isolated from apparently healthy wild living small rodents in Denmark (4) Each of thes isolates were agglutinated by *Y enterocolitica* O antisera 4–33 and 21 and by *Y pseudotuberculosis* O antiserum IIB

A total of eight strains adhered firmly to the cel surface and to the glass Seven of these strains belonged to O serogroup 4/biotype 1 (Wauters biotype scheme) isolated from small rodents It was not possible to determine the invasiveness of these strains owing to the great number of bacteria attached extracellularily The phenomenon was reproducible on several repeated examinations Additional washing with PBS was not sufficient to remove the bacteria

Human Strains

All of 22 strains belonging to O serogroup 3/ biotype 4 from human patie with gastroenteritis

ıBLE 1 *Interaction with HeLa Cells among* Y *erocolitica and* Y enterocolitica *Like Bacteria Isolated from Environmental Sources in Scandinavia*

ogroup[a]	No of strains tested	No invasive	No adhesive
	9	–	–
	1	1	–
	7	–	–
	35	–	7
	2	–	–
	41	–	–
	5	–	–
	8	–	–
	11	–	–
	5	–	–
	10	–	–
	1	–	–
	13	–	–
	9	–	–
	12	–	–
	23	3	–
;	130	–	1
	5	–	–
	4	–	–
l	331	4	8

abic figures indicate antigenical relationship to O roups of *Y enterocolitica* Roman figures O roups of *Y pseudotuberculosis*
ixed (agglutinated by 2–5 of the antisera 4 16 18 12 1A 1B and IIB in ten different combinations)
r non agglutinable

were invasive (Table 2) There was a tendency for these strains to adhere extracellularly Detachment of HeLa cells from the coverslips was also observed In contrast, seven strains of O serogroup 3 isolated from small mammals and water, were non invasive (Table 1) None of these strains could be ascribed to biotype 4

Another six isolates from human patients, belonging to other O serogroups, did not interact with the HeLa cells However, the clinical significance of these strains has not been confirmed Further, neither invasion nor adhesion was discovered among 20 strains isolated from healthy humans

Reference Strains

A total of 15 (45%) of 33 reference strains representing *Y enterocolitica* O-serogroup 1–34 were invasive (Table 3) Invasiveness was also indicated for six of the seven reference strains of *Y pseudotuberculosis*

Relationship between HeLa Cell Interaction and Enterotoxin Production

A close correlation between invasiveness and enterotoxigenicity was demonstrated for human clinical isolates belonging to O-serogroup 3/biotype 4 All 22 isolates were invasive, and 20 of these also produced enterotoxin at 22 °C

Interaction with HeLa cells was *not shown* for any of the 64 environmental strains which were enterotoxigenic

Three reference strains were both invasive and enterotoxigenic (O-serogroup 5–27 20 and 22)

LE 2 *Interaction with HeLa Cells among* Y enterocolitica *and* Y enterocolitica *Like Bacteria Isolated from Healthy and Diseased Humans in Norway*

rogroup	Healthy humans		Diseased humans	
	No tested	No invasive	No tested	No invasive
	–	–	22	22
	1	–	1	–
	2	–	1	–
	5	–	1	–
)	1	–	–	–
	4	–	–	–
	–	–	1	–
G[a]	1	–	1	–
	6	–	1	–
d	20	–	28	22

n agglutinable

TABLE 3 *HeLa Cell Invasiveness and Enterotoxigenicity among Reference Strains of* Y enterocolitica *[illegible]* Y pseudotuberculosis

O serogroup	Strain[a]	Source	Invasive[b]	Enterotoxin
Y enterocolitica				
1	IP 6	Chinchilla	+	−
2	IP 8	Hare	+	−
3	IP 85	Swine	(+)	−
4–32	IP 96	Chinchilla	+	−
4–33	IP 1476	Water	−	−
5	IP 123	Cow	−	+
5–27	IP 47	Monkey	+	−
5–27	IP 885	Dog	+	+
6–30	IP 102	Human	−	−
6–31	IP 1477	Water	−	−
7– 8	IP 106	Guinea pig	−	−
8	IP 107	Human	+	−
8	P 311	Human	+	−
8–19	Fy 50	?	−	−
8–19	IP 842	Human	−	−
9	IP 201	Human	+	−
10	IP 551	Human	(+)	−
11–23	IP 105	Human	−	−
11–24	IP 841	Human	−	−
12–25	IP 490	Hare	−	+ +
12–26	IP 103	Sheep	−	+
13– 7	IP 553	Human	−	−
14	IP 480	Human	−	+
15	IP 614	Human	+	−
16	IP 1475	Water	−	−
16–29	IP 867	Human	−	−
17	IP 955	Water	−	−
18	IP 846	Human	(+)	−
20	IP 845	Human	+	+
21	IP 1110	Human	+	−
22	IP 1367	Human	+	+
28	IP 1474	Water	−	+ +
34	IP 1501	Human	−	−
Y pseudotuberculosis				
IA	402	?	+	−
IB	403	?	+	−
IIA	407	?	−	−
IIB	401	?	+	−
III	405	?	+	−
IV	404	?	+	−
V	406	?	+	−

[a] Strains with the IP prefix were received from Institut Pasteur Paris The other reference strains were obtained fr[illegible] Dr *Winblad* Malmø

[b] + = positive reaction − = negative reaction (+) = weak reaction + + − enterotoxin production both at 22 and 37 °C

DISCUSSION

Most of *Y enterocolitica* and *Y enterocolitica* like bacteria from environmental and human sources in Scandinavia were non invasive in the HeLa cell model employed in this study Invasiveness was indicated for all strains otherwise recognized pathogenic and furthermore for three strains rela[illegible] to *Y pseudotuberculosis* HeLa cell invasiveness [illegible] documented feature of *Y pseudotuberculosis* [illegible] These results are in a[illegible]ance with the d[illegible]

ed from North America by *Lee et al* (6) and Japan by *Une et al* (10)

et al (6) suggested that strains that cannot penetrate HeLa cells which have a very weak r against bacterial infection are most prob not able to invade epithelial cells *in vivo* On her hand invasiveness may be more prevalent g *Y enterocolitica* and *Y enterocolitica* like a than indicated by laboratory models eness in *Y enterocolitica* is mediated by a id (3 11) Such a factor may be lost after uring of the strains Further 131 of the s examined were isolated from wild living mammals which were trapped by methods elect active individuals and thereby reduce the bility of discovering diseased animals This may give a false impression of the prevalence of ne strains

La cell invasiveness was demonstrated for nce strains with known pathogenicity for man mals (O-serogroup 1 2 3 5–27 8 9) On the hand, some strains belonging to O serogroups uncertain clinical significance were also ne (O serogroup 4–32 10 15 18 20 21 Similar observations were reported by *Pedersen* (8) However these reference strains have been cted to repeated subculturing and prolonged ge which may have influenced both their siveness and enterotoxigenicity

ncly considered invasion of HeLa cells could useful but not absolute criterion of the ogenicity of *Y enterocolitica* isolates *Une* (9) ested that HeLa cell infectivity is not sufficient luate the pathogenicity of these bacteria This or emphasized that primarily the ability to trate epithelial linings and secondarily the ty to survive or multiply within macrophage cells seems to be required to establish infection *Y enterocolitica in vivo* Further some kind of sion may be necessary to initiate invasion The rved adhesion phenomenon may represent the al stage in the invasion process Pathogenicity in nterocolitica and *Y enterocolitica* like bacteria also be ascribed to enterotoxin production (5 However adhesion colonization or receptor ors may be needed to achieve clinical effect of *Y enterocolitica* enterotoxin analogous to the nization factors required by enterotoxigenic *herichia coli* (2)

oth invasiveness and enterotoxin production urred with very high frequency among human ical isolates of *Y enterocolitica* O-serogroup 3 invasiveness study of *Une et al* (10) and the y on enterotoxigenicity of *Pai et al* (7) indicate that a corresponding relationship likewise exists for human clinical isolates of O-serogroup 5 8 and 9 Possibly these two pathogenicity factors act in concert to enhance the ability of *Y enterocolitica* to provoke enteric disease

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The valuable discussion of *Bjorn Peter Berdal Gabriel Ånestad Jean Michel Alonso* and *Enok Tjøtta* is gratefully acknowledged

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QUANTITATION OF M ANTIGEN IN LANCEFIELD EXTRACTS OF GROUP A STREPTOCOCCI TYPE 12 USING ELECTRO-IMMUNO ASSAY

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Eliasson, I Svensson M L Ramstorp G Schalen C & Christensen P Quantitation of M antigen in Lancefield extracts of group A streptococci type 12 using electro immuno assay Acta path microbiol scand Sect B 88 299–302 1980

Anti type 12 serum incorporated in agarose polyethylene glycol gel in a concentration of 1 5% (vol/vol) was found to enable a distinct »rocket« precipitate in electro immuno assay using hot hydrochloric acid extract of type 12 group A streptococci This precipitate was removed by trypsin treatment of the extract and on addition of anti M12 typing serum but not of five other typing sera to the extract. The streptococcal component responsible for this precipitate was eluted from a CM-cellulose ion exchange column at pH 6 5 These findings demonstrated that the precipitate was caused by the M12 antigen Crossed immuno-electrophoresis of hot hydrochloric acid extracts of three different type 12 group A streptococci showed that the electrophoretic mobility of the M12 antigens was similar in the three extracts A linear correlation was obtained between the concentration of the M12 antigen and the height of the precipitate obtained in the electro immuno assay using different dilutions of a standard type 12 extract. M12 antigen could thus be quantitated by the electro immuno assay In quantitation experiments uniformly prepared extracts of five randomlyselected freshly isolated type 12 strains were found to contain from 130 to 1850% of M12 antigen respectively (expressed in % of the content of the standard type 12 extract)

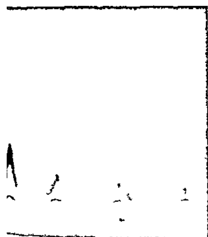
Key words Streptococci Lancefield extract M protein quantitation electro-immuno assay

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The M antigens are important virulence factors in ..p A streptococci (6 9 14) However few ..hods have been devised for quantitation of M ..gens in streptococcal preparations *Becker et al* ..used a *Mancini* technique for quantitation of M ..gen in *Lancefield* extracts To our knowledge all ..er immuno-chemical procedures hitherto descri..d i.e modifications of the capillary precipitin ..thod originally used for typing of group A ..ptococci (8) These modifications comprise ..mation of the *precipitate* obtained between a ..cefield extract and the corresponding type ..cific rabbit serum from + to ++++ (14) or ..asurement of the precipitate as in the *Oudin* ..chnique (4 12)

The present paper concerns quantitation of M antigen in *Lancefield* extracts of type 12 group A streptococci using electro immunoassay Measurement of M antigen in the *bacteria* with this method or any of the procedures described above involves cultivation extraction and quantitation of the antigen present in the extract Variation in quantitative results may originate from all these three steps The aim of this investigation was primarily to describe a simple and exact method for quantitation of the M antigen content in *Lancefield* extracts however the possibility to quantitate the M12 antigen per weight unit of bacteria was also considered



f Electro immuno assay of a standard extract of
to
4

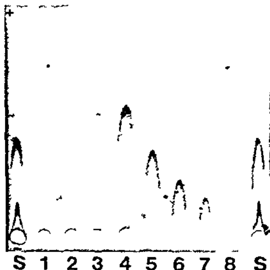


Fig 2 Quantitation of the M protein content in a Lancefield extract of a freshly isolated type 12 group A streptococcus using electro immuno assay S Undiluted standard extract 1–8 Twofold dilutions up to 1 128 of the extract to be examined The M protein content of the extract was calculated to 1430% of the standard extract content.

pitate was caused by the M12 antigen Addi of crude IgG Fc receptor from type 15 group A tococci (3) in equal volumes to the standard ct did not affect the expected height of the pitate

examine the accuracy of the electro immuno- system designed as above for the quantitation protein in *Lancefield* extracts the undiluted ard extract was applied in 19 wells on each of different plates run on different occasions The assay« variation was 2 9–3 4% (SD) and the assay« variation of the mean heights from plate was 1 2% (SD) The standard diluted in M steps from 1 2 to 1 32 gave equally ducible results

parison of Electrophoretic Mobility of M antigen ancefield Extracts of Three Different Type 12 s

e extracts of three strains 1800 300 and 305 tested in crossed immuno-electrophoresis extract gave a precipitate situated anodally in on to the application well The electrophoretic y of the component responsible for the major pitate was similar in all three extracts These pitates did not form after addition of Ca-serum al volumes to the extracts

of Lancefield Extracts from Type 12 Group A tococci Isolated from Clinical Specimens

x hydrochloric acid extracts of five freshly ed type 12 group A streptococci were tested in electro-immuno assay A minor precipitate ar to that given by the trypsin resistant component in the standard was not obtained with any of these strains It may be noted that the freshly isolated strains were not treated with enzyme inhibitors prior to extraction in contrast to the standard (see above) For quantitation of M12 antigen each precipitating extract was diluted to give a »rocket« of a height between that obtained with undiluted and that obtained with 1 4 diluted standard extract (Fig 2) The heights of the precipitates obtained with the different dilutions of the standard extract were plotted against the standard extract concentrations in a diagram after which the M12 antigen content at a suitable dilution of the extract under test could be calculated The M12 antigen contents of the five extracts were 130 740 1430 1600 and 1850% respectively of the standard extract content

Extracts were made concomitantly of two different strains each cultured in five bottles of 300 ml Todd Hewitt broth (from the same batch of broth) The M protein content in the extracts from one strain varied between 66 and 100% (mean 85%) and in the extracts of the other strain between 104 and 200% (mean 138%) of the standard extract content

DISCUSSION

The M antigen in *Lancefield* extracts of type 12 group A streptococci gave distinct »rocket precipi

MATERIALS AND METHODS

Bacterial Strains and Extracts

Group A streptococci type 12 were kindly supplied by Dr *Burova* Leningrad (strain 1800) and Dr *Sramek*, Prague (strains 300 and 305) Five freshly-isolated group A streptococcal strains belonging to M-type 12 were randomly selected from among the strains sent to our department Hot hydrochloric acid extracts (8) were prepared from 18-hour cultures at 37 °C on 300 ml Todd Hewitt broth After culturing, the streptococci were washed three times in distilled water and lyophilized 0 10 g of the powder was then suspended in 0 15 M saline and the pH was adjusted to 2 0 with 0 1 M HCl After heating in a boiling water bath for 10 min, the pH was neutralized to 7 0 using 0 1 M NaOH and the suspension centrifuged at 3000 *g* for 30 min The volume of each extract was then adjusted to 3 0 ml with saline

A hot hydrochloric acid extract of strain 1800 prepared from a 20-litre Todd Hewitt broth culture (13), with the addition of benzamidine chloride and iodo acetic acid to 10 mM during washings and extraction procedures, was used as a standard preparation through out the study and stored at −20 °C until use

Rabbit Sera

Two different anti type 12 sera, Lu and Ca, were used The latter serum and anti-type 4, 28, 48 and 60 sera were M-typing sera, kindly supplied by Dr *El Kholy* Cairo Unless otherwise indicated, the unabsorbed Lu serum was used as anti-type 12 serum

Electro-immuno Assay

Electro-immuno assay (11) was used for quantitation of M antigen in the extracts Twenty ml 0 6% (w/vol) agarose (electroendosmosis ($-m_r$) 0 16–0 19, Sea Kem Rockland, ME USA) in Tris/barbital buffer (0 07 M Tris and 0 02 M sodium barbital) containing 0 3 mM calcium lactate and 2 mM sodium azide, was mixed with various volumes of anti type 12 serum and polyethyleneglycol 6000 (Kebo Sweden) (PEG) and poured in a 1 mm thick layer over a 205 × 110 mm glass plate Circular wells taking 5 µl extract were cut 80 mm from the projected anode A current of 3–4 V/cm was applied to the plate The extract to be tested was then placed in the wells and the current was increased to 6 V/cm for 16 hours The plates were washed in 0 9% NaCl and stained with Coomassie Blue

Crossed Immuno-electrophoresis

Crossed immuno electrophoresis (10) was performed using gel and buffer-systems as described above The extract was first subjected to electrophoresis (20 V/cm for 40 min) in agarose without antiserum or PEG Agar slips, 60 × 3 mm, were then cut out from the gel containing the separated extract, in the direction of the current and the slip was placed edge to edge with an agarose gel containing 1 5% anti-type 12 serum and 3% PEG The extract components were forced into the antiserum-containing gel by application of a current of 6 V/cm for 16 hours (at an angle of 90° to the first electrophoresis) Washings and staining were perfor as described above

Crude IgG Fc-Receptor Preparation

A crude preparation of IgG Fc receptor from type group A streptococci was obtained from a prepara electrophoresis as described (3) This preparation aggl nated Ripley-sensitized human red cells when dilu 1 20 000 or less (3)

Ion Exchange Chromatography

Ion exchange chromatography of hot hydrochl acid extracts of type 12 group A streptococci performed on CM-cellulose (Pharmacia Fine Chemic Uppsala, Sweden) as described by *Fox* (5)

RESULTS

Identification of M12-Antigen Precipitate in Electi immuno Assay

Anti-type 12 serum was incorporated in t agarose gel in concentrations from 1 to 3% (vo vol) and the PEG concentration was varied betwe 0 and 2% (w/vol) It was found that 1 5% seru and 2% PEG enabled a maximally distinct »rocke precipitate, when undiluted type 12 extract standa preparation was tested in the electro-immuno assa The results were similar without the addition PEG but the precipitates obtained were considerab less sharp Electrophoresis at a voltage of 20 V/c for 4 hours gave less distinct precipitates than tho obtained at a voltage of 6 V/cm for 16 hours At V/cm, the precipitate formed extended about 1 mm from the application well (for accuracy, s below) Varying the dilution of the standard extra up to 1 4 resulted in a linear correlation between tl concentration of the extract and the height of tl major precipitate (Fig 1) When using the standar extract, an additional precipitate was forme extending about 3 mm anodally from the applica tion well

In the following a concentration of 1 5% ant type 12 serum and 2% PEG was used Undilute standard extract as well as 1 2 and 1 4 dilution were included in each electro-immuno assay

The major precipitate disappeared upon trypsini zation of the standard extract (for procedure, see re 3) while the minor precipitate was unaltered Testing of various fractions obtained from ion exchange chromatography of the standard extrac showed that the components responsible for the major precipitate were eluted from the CM-cellulose column at pH 6 5 Formation of the major precipitate was completely inhibited on addition of anti-type 12 typing serum (Ca) in equal volumes to the standard extract but not by addition of anti-4, 28, 48 or 60 M-typing sera Thus, the major

Acta path microbiol scand Sect B 88 303–306 1980

YERSINIA ENTEROCOLITICA AND *Y ENTEROCOLITICA*-LIKE BACTERIA ISOLATED FROM HEALTHY HUMANS IN NORWAY

GEORG KAPPERUD

Norwegian Defence Microbiological Laboratory Oslo and Zoological Institute University of Oslo Norway

Kapperud, G *Yersinia enterocolitica* and *Y enterocolitica* like bacteria from healthy humans in Norway Acta path microbiol scand Sect B *88* 303–306 1980

Faeces samples from a total of 397 presumably healthy students in Norway were collected during April to September *1979* Twenty strains of *Y enterocolitica* and *Y enterocolitica* like bacteria were isolated from *10* (5%) of these students Six students harboured 2–4 antigenically distinct strains Of the total of 20 strains 14 were typical *Y enterocolitica* isolates representing five different serogroups (4 5 6 7 8 13–7 and non agglutinable) The remaining six strains were tentatively designated »Y enterocolitica like bacteria« They showed atypical reactions with respect to one or more of the following characters fermentation of rhamnose sucrose cellobiose and sorbose The strains recovered from healthy humans in this work were similar to strains previously isolated from wild living mammals, fish and water in Scandinavia Nine strains produced enterotoxin at 22 °C but not at 37 °C

Key words *Yersinia enterocolitica* humans healthy carriers

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Yersinia enterocolitica and *Y enterocolitica* like bacteria are often present in nature (15) This vast reservoir may be a considerable source of contamination for humans through drinking water food and animal contacts Most of the strains prevailing in this reservoir are antigenically and biochemically differ from those associated with the typical clinical manifestations of *Y enterocolitica* infections However there has been an increased awareness of the possible role of such »environmental« strains in atypical clinical syndromes (15)

Numerous investigations have documented the presence of *Y enterocolitica* serogroup 3 and other serogroups with recognized clinical importance among different categories of human patients However little work has been done to evaluate the human normal flora of *Y enterocolitica* and *Y enterocolitica* like bacteria (1 2 7) The present study was undertaken to survey the presence of these bacteria in stool specimens from presumably healthy students in Norway

MATERIALS AND METHODS

Collection of Faeces Samples

During the period April to September 1979 a total of 397 faeces samples were collected from six different groups (A–F) of presumably healthy students (age group 18–30 years) in Norway (Table 1) Faeces samples were collected on swabs and stored on a modified Stuart transport medium (16)

A In the middle of April 18 samples were collected from high school students attending a winter camp in Hallingdal central Norway The drinking water was untreated in this area

B During the last week of April faeces samples were collected from 108 students of biology University of

tates in the electro immuno assay with unabsorbed anti type 12 serum included to the gel This finding made it possible to develop a method for the quantitation of M12 antigen using electro immuno assay

Recent investigations indicate that M antigens are composed of repeating amino acid sequences (1) M antigens purified from hot hydrochloric acid extracts of all types investigated including type 12 group A streptococci show a marked heterogeneity in polyacrylamide electrophoresis (5 6 7) This property attributable to differing molecular weights of the M antigen fragments obtained by hot acid extraction has been called the »multiple banding phenomenon« each band so obtained contains compounds which are antigenetically identical making possible quantitation by immunochemical methods (5) The occurrence of multiple bands in polyacrylamide electrophoresis of M antigens does not imply that the bands are distributed »by chance« i e varying from one occasion to another M proteins from different batches of cells of the same strain and also from unrelated strains of the same serotype have nearly identical patterns of mobility and each serotype has its characteristic distribution of bands (5) In the present paper crossed immuno-electrophoresis – which separates the antigens according to electrophoretic mobility – of three extracts of different type 12 group A streptococci gave a symmetrical precipitation peak in all experiments indicating a homogeneous electrophoretic mobility of the type 12 antigen fragments in *Lancefield* extracts This conclusion was also supported by the fact that the bulk o M antigen from a hot hydrochloric acid extract was eluted from CM-cellulose in one major peak in agreement with the findings of *Fox & Wittner* (7) The homogeneous electrophoretic mobility of M12 antigens in *Lancefield* extracts indicated that the outcome of an electro immuno assay in which the extract of one strain is compared with the extract of another strain would not be erroneously influenced by differences in electrophoretic behaviour

Like all other applications of the electro immuno assay technique the procedure described in the present paper is a simple quick and reproducible way of determining individual components in a complex mixture A greater sensitivity than with the capillary precipitin methods is probably achieved Furthermore the qualitative estimation of precipitates makes it possible to avoid confusion with the other components present in a crude bacterial extract It should be pointed out however that the amount of extractable M antigen does not necessarily reflect the amount present i bacteria Thus the extent to which the electr immuno assay can be used for comparison of th antigen quantities in different type 12 strain depends on standardization of the cultivation an extraction procedures These problems are currentl being analyzed

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Faeces samples from a total of 397 presumably healthy students in Norway were collected during April to September 1979 Twenty strains of *Y enterocolitica* and *Y enterocolitica* like bacteria were isolated from 10 (3%) of these students Six students harboured 2–4 antigenically distinct strains Of the total of 20 strains 14 were typical *Y enterocolitica* isolates representing five different serogroups (4 5 … 7–8 13–7 and non agglutinable) The remaining six strains were tentatively designated › *Y enterocolitica* like bacteria‹ They showed atypical reactions with respect to one or more of the following characters fermentation of rhamnose sucrose cellobiose and sorbose The strains recovered from healthy humans in this work were similar to strains previously isolated from wild living mammals fish and water in Scandinavia Nine strains produced enterotoxin at 22 °C but not at 37 °C

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…rsinia enterocolitica and *Y enterocolitica* like …ria are omnipresent in nature (15) This vast …rvoir may be a considerable source of contami…on for humans through drinking water food …animal contacts Most of the strains prevailing …his reservoir are antigenically and biochemically …inct from those associated with the typical …cal manifestations of *Y enterocolitica* infec…s However there has been an increased …reness of the possible role of such ›environmen… strains in atypical clinical syndromes (15) …Numerous investigations have documented the …sence of *Y enterocolitica* serogroup 3 and other …groups with recognized clinical importance …ong different categories of human patients …wever little work has been done to evaluate the …an normal flora of *Y enterocolitica* and *Y …erocolitica* like bacteria (1 … 7) The present study was undertaken to survey the presence of these bacteria in stool specimens from presumably healthy students in Norway

MATERIALS AND METHODS

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A In the middle of April 18 samples were collected from high school students attending a winter camp in Hallingdal central Norway The drinking water was untreated in this area

B During the last week of April faeces samples were collected from 108 students of biology University of

Oslo one week after their Easter vacation Most of the students spent these holidays in non urban areas with untreated drinking water

C During the first two weeks of May, faeces were sampled from 72 medical students, University of Oslo The samples from group B and C were obtained during the spring thaw in southern Norway

D In June, 40 samples were obtained from zoologists attending field courses

E The August samples were collected from 77 medical students University of Bergen, the week subsequent to their summer vacation During the summer, the students lived at widespread localities all over southern Norway Most of the students had been exposed to untreated drinking water through mountain hiking and other outdoor activities

F In September, 82 samples were collected from students at the Veterinary College of Norway, Oslo

Processing Collected Material

Cultivation from the swabs was performed within one week on lactose/bromothymol-blue (Drigalski) agar and lactose/sucrose/ urea (LSU) agar The agar plates were incubated at room temperature (approximately 22 °C) for 48 hours Sub cultures were made on lactose agar of all colonies showing morphological similarity to *Y enterocolitica* Urease positive strains were selected and a primary biochemical characterization was performed by the »three-tube method« described by *Lassen* (14) If the results indicated *Yersinia* the strains were subjected to further biochemical and antigenic characterization as specified previously (8)

Strains isolated from the same faeces sample were selected for study if they were antigenically and/or biochemically different

RESULTS

Altogether, 20 strains of *Yersinia enterocolitica* and *Y enterocolitica*-like bacteria were isolated from 10 (3 %) of the 397 faeces samples from presumably healthy students in Norway (Table 1) The antigenic and biochemical characteristics of the isolates are presented in Table 2

Characterization of the Isolates

Among the 20 strains isolated 14 were typical *Y enterocolitica* isolates (Table 2) Antigenically these strains were related to five different serogroups One strain was non-agglutinable Eleven of the *Y enterocolitica* isolates could be ascribed to Wauters biotype 1 (18) The remaining three strains differed

They showed atypical reactions with respect to one or more of the following characters fermentation of rhamnose, sucrose, cellobiose and sorbose In the absence of a generally accepted taxonomy, these

TABLE 1 *Isolation of* Y enterocolitica *and* enterocolitica-*Like Bacteria from Six Different Grou[ps of] Healthy Students in Norway*

Group	Date	No examined	Individuals harbou[ring] *Yersinia* strains No	%
A	April	18	–	–
B	April	108	6	6
C	May	72	2	3
D	June	40	2	5
E	August	77	–	–
F	September	82	–	–
Total		397	10	3

TABLE 2 *Antigenic and Biochemical Characteriza[tion of]* Y enterocolitica *and* Y enterocolitica-*Like Bacteria f[rom] Healthy Students in Norway*

Serogroup[a]	Biotype[b]	No of str[ains]
Y enterocolitica		
4	1	1[c]
5	1	2[d]
6	1	2[d]
6	1 X–	2
6	1 VP–	1
7–8	1	1[c]
13–7	1	2
13–7	1 VP–	2[c]
NAG	1 X–	1[c]
Y enterocolitica-like bacteria		
16	So– VP–	1[c]
NAG	R +	1
NAG	S– VP–	2
NAG	C– So– VP–	1
NAG	R + S– C– VP–	1
Total		20

a) NAG = non agglutinable
b) According to Wauters biotype scheme (18)
c) = Enterotoxin production at 22 °C (10)
d) = Both strains produce enterotoxin at 22 °C

Key to the symbols
X– xylose non fermenting
VP– Voges Proskauer negative (at 22 °C and 37 °C)
So– = sorbose non fermenting
R + rhamnose fermenting
S– = sucrose non fermenting
C– = cellobiose non fermenting

ns were tentatively designated »Y enterocoli like bacteria«

nage of Several Distinct Strains

our of the ten students carrying *Yersinia* strains boured two strains with distinct antigenic erties One person harboured three distinguish strains In a sixth case four antigenically rent strains were isolated from the same faeces ple, namely serogroup 6 4 7–8 and non utinable

ne person harbouring *Y enterocolitica* sero-p 6 in April was asked to take a second sample weeks later Two antigenically distinct strains group 16 and non agglutinable) were isolated n this second sample The original serogroup 6 not detected No isolations were achieved from rd sample taken in June

ical Significance

our of the ten persons harbouring *Yersinia* ns claimed that they suffered from recurrent rhoea and abdominal pain These symptoms ever could well be explained by psychical stress ms)

DISCUSSION

examined students harboured *Yersinia entero ica* and *Y enterocolitica* like bacteria which antigenically and biochemically similar to ins previously isolated from wild living mam fish and water in Scandinavia (8 9 12 13) ie students harboured several distinct strains logous results were obtained by *Alonso et al* (1) reported that humans inhabiting a rural area in ice were healthy carriers of strains similar to e isolated from wild living small mammals in same area

he duration of the carriage was not determined s possible that the isolations may reflect a sient passage of the bacteria through the tinal tract rather than a more permanent carrier

lost of the students had been exposed to eated drinking water This may be an important e of contamination (13) Altogether 18 of the isolations now reported were made during the ng thaw when bacterial contamination of the king water is increased However the results not justify any statistically significant conclu s concerning a possible seasonal variation in the age of *Yersinia* strains Seasonal fluctuations in frequency of human yersiniosis with a peak ng the cold months of the year have been orted from some European countries (3) A similar trend was observed in an earlier study concerning the incidence of *Yersinia* strains in fish and water in Norway (12)

No clinical effect of the isolated strains could be established with certainty Obviously examination of a very large material would be required to determine a possible clinical or sub-clinical role of the strains in question When *Y enterocolitica* or *Y enterocolitica* like bacteria are isolated from diseased individuals the possibility of a causal connection should be examined critically The isolation might well reflect an incidental contamination from the extensive environmental reservoir of these bacteria having no causal connection with the disease

Nine of the twenty strains isolated in this work produced enterotoxin at 22 °C but not at 37 °C (10) Conclusive results have not yet been obtained concerning the clinical significance of the *Y enterocolitica* enterotoxin Furthermore none of the isolates were invasive in a HeLa cell model (11)

Serogroup 3/biotype 4 and serogroup 9/biotype 2 responsible for the typical clinical manifestations of *Y enterocolitica* infections in Europe (15) were not recovered in this work Nevertheless strains similar to those isolated have occasionally been reported in connection with atypical clinical syndromes (4 5 6 7 15 17) The presence of healthy carriers of such strains may be a potential hazard for susceptible hosts for example immunologically deficient individuals through direct or indirect human transmission

I want to thank the 397 students who contributed to the fulfilment of this study

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IN VITRO ANTIBIOTIC SENSITIVITY TESTING OF *VIBRIO ALGINOLYTICUS*

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Larsen J L & Farid A F *In vitro* antibiotic sensitivity testing of *Vibrio alginolyticus* Acta path microbiol scand Sect B 88 307–310 1980

V alginolyticus from environmental sources of different geographical areas and some human pathogenic strains were investigated for their sensitivity to 29 different antimicrobial agents using the agar diffusion method All strains were sensitive to Gentamicin Neomycin Sulfaisodimidin Sulfamethoxazole Trimethoprim Rifamycin Nalidixan and Linco Spectin and all except one to Trimethoprim Tetracycline Chloramphenicol Nitrofurantoin and Tobramycin Between 80 and 94% of tested strains were sensitive to Clindamycin Cephalotin Kanamycin Tylosin and Spiramycin while Ampicillin, Vancomycin and Novobiocin were effective to approximately 50% A high percentage of resistance was found to Meticillin Carbenicillin and Lincomycin All strains were resistant to Penicillin and Fucidin No variation in sensitivity pattern between environmental strains isolated from different areas was detected Great correlation was found among environmental and human pathogenic strains which could be helpful as guidance in therapy

Key words *Vibrio alginolyticus* antibiotic sensitivity testing *in vitro*

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The halophilic bacterium *Vibrio alginolyticus* urs in marine coastal waters with highest valence during the bathing season when water perature is high (4 6 8 10 12 16) It has ently been recognized as a human pathogen and lated from infection of the ear eye nose head tum and bronchial washings finger hand and st, leg foot and other unspecified sites of ients exposed to marine environments (2 4 8) 12 13) The infections are occasionally nplicated by septicaemia

Despite the increasing importance of *V alginolyticus* from a medical point of view strains isolated m environmental sources are very seldom tested r their anti microbial susceptibility (5) Strains lated from human septic infections are always estigated for their susceptibility to a number of rious antibiotic agents Generally *V alginolyticus* are certainly not extensively tested for anti microbial susceptibility in the world literature and no such investigation has been carried out in Denmark

This paper presents the results of determinations performed on well-characterized *V alginolyticus* strains isolated from different environmental sources in different geographical areas on the Danish coast Some human pathogenic strains collected from different sources were tested under the same circumstances and the results were compared

MATERIALS AND METHODS

Strains of *V alginolyticus* from environmental sources comprised thirty marine strains fifteen estuarine and four sediment isolates (*Larsen et al* (7) as well as 2 other sediment Vibrio strains These strains were previously isolated from different bathing areas on the Danish

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TABLE 1 *Number and Percentage of Sensitive (Highly Sensitive +++ and Moderately Sensitive ++) VAE and VAH strains*

Antimicrob al/ug[a]		VAE 51 strains		VAH 7 strains		Antimicrobial/ug[a]		VAE 51 strains		VAH 7 strains	
		No of sens	%	No of sens	%			No of sens	%	No of sens	%
[illegible][a]	10	0	0 0	0	0 0	Chloramphenicol	30	50	98 04	7	100 00
[illegible] High[b] [illegible]	100	19	37 25	2	28 57	Rifamycin[c]	30	51	100 00	7	100 00
[illegible]	10	24	47 06	3	42 86	Erythromycin	15	40	78 43	7	100 00
	10	5	9 80	0	0 00	Lincomycin	15	11	21 57	1	14 29
[illegible]cillin	100	13	25 49	1	14 29	Fucidin	50	0	0 00	0	0 00
[illegible]	30	47	92 16	7	100 00	Vancomycin	30	29	56 86	5	71 43
[illegible]	30	34	66 67	5	71 43	Nitrofurantoin	30	50	98 04	7	100 00
[illegible]	30	42	82 35	6	85 71	Nalidixan	30	51	100 00	7	100 00
[illegible]	30	51	100 00	7	100 00	Linco Spectin[d]		51	100 00	7	100 00
[illegible][c]	120	51	100 00	7	100 00	Tylosin[c]	150	38	74 81	6	85 71
[illegible]	30	35	68 63	5	71 43	Novobiocin[c]	100	24	47 06	6	85 71
[illegible]sod m d n	250	51	100 00	7	100 00	Spiramycin[c]	200	40	78 43	7	100 00
[illegible]methoxazole [illegible]ethoprim	25	51	100 00	7	100 00	Clindamycin[c]	25	48	94 12	5	71 43
[illegible]thoprim[c]	5 2	50	98 04	7	100 00	Tobramycin[c]	40	50	98 04	7	100 00
[illegible]cycl ne	30	50	98 04	7	100 00						

[a] ...biotic concentration per disc
[b] ...ncentration in I U
[c] ...sco discs
[d] ...mpound of Spectomycin 200 μg + Lincomycin 19 μg

...Nalidixan and Linco-Spectin whereas they are resistant to Penicillin and Fucidin

Actually strains with intermediate susceptibility comprised those originally evaluated as moderately sensitive (++) and slightly sensitive (+) When both the highly sensitive (+++) and moderately sensitive (++) strains are considered sensitive their total number and percentage are as given in Table 1 The results of the Table added Gentamicin and Rifamycin to the effective group of antibiotics shown in the figure as all VAE and VAH were sensitive Moreover 98% of VAE and all VAH were sensitive to Trimethoprim Tetracycline Chloramphenicol Nitrofurantoin and Tobramycin Ninety four per cent and ninety two per cent of VAE and seventy-one per cent and all VAH were sensitive to Clindamycin and Cephalotin respectively

Another [illegible] the str[illegible] and S[illegible]

[illegible]novobiocin were effective against approximately fifty per cent of strains tested Actually all strains were highly sensitive to Sulfaisodimidin This sulphonamid is not presented in Fig 1

DISCUSSION

A correct strategic and tactic use of chemotherapy against bacterial infections requires reliable information on the efficiency of drugs against the causal bacterial species Such data are gained from bacteriological laboratories performing antibiotic sensitivity testing (11) The preliminary or basic knowledge about the action of different antimicrobial agents on the species of bacteria encountered is greatly helpful No such data are available in case of the halophilic bacterium *Vibrio alginolyticus* as it is a relatively new species considered pathogenic to man

Twenty nine different antimicrobial agents were used to investigate the susceptibility of *V alginolyticus* strains The *in vitro* testing was performed by the agar diffusion method which in a recent study (5) proved to be applicable for this group of organisms

The present study demonstrates a high incidence of sensitivity to different agents All strains of VAE and VAH were sensitive to Gentamicin Neomycin Sulfaisodimidin Sulfamethoxazole Trimethoprim Rifamycin Nalidixan and Linco-Spectin All strains

coasts and were characterized by their biochemical behaviour (7) The human pathogenic strains were obtained from Dr *D G Hollis* CDC, Atlanta, Georgia, U S A (strains 2300/79, 1424/79, and 1586/79), Dr *Mary L Fried*, Tucson Medical Center, Tucson, Arizona, U S A (T M C strain) and Dr *A von Graevenitz*, Yale University School of Medicine New Haven Connecticut, U S A (Yale strain) The last two strains were previously described (*15*, *17* respectively) Two human pathogenic strains previously isolated in Denmark (9) were also examined Unless otherwise indicated, *V alginolyticus* strains from environmental sources are called VAE, whereas human pathogenic strains are called VAH

The antibiotic discs or tablets of 29 anti-microbial agents used in this study are commercially available (AB-Biodisk, Solna, Sweden, and A/S Rosco, Neo Sentitabs®, Taastrup, Denmark) and are listed in Table 1

Anti-microbial sensitivity was determined by the standard agar diffusion method (3) though the organisms were grown in trypticase soy broth (BBL) with 1% sodium chloride added and the cultures were applied to Mueller-Hinton agar (Difco) plates with addition sodium chloride (0 5%), for susceptibility testing

Interpretation of results was performed by measurin the diameters of the inhibition zones in millimeters, an the readings were compared with corresponding manu facturing tables Mostly four grades were evaluatec highly sensitive (+++), moderately sensitive (++ slightly sensitive (+), and resistant

RESULTS

The results are presented in Fig 1, illustrating th zone diameters and accepted breakpoints betwee resistance and susceptibility for all antibiotics used Strains giving inhibition zones that could not b evaluated as highly sensitive were considere intermediately susceptible and presented in the are between the solid and broken lines The figur shows that all VAE and VAH strains are sensitiv to Neomycin, Sulphamethoxazole-Trimethoprim

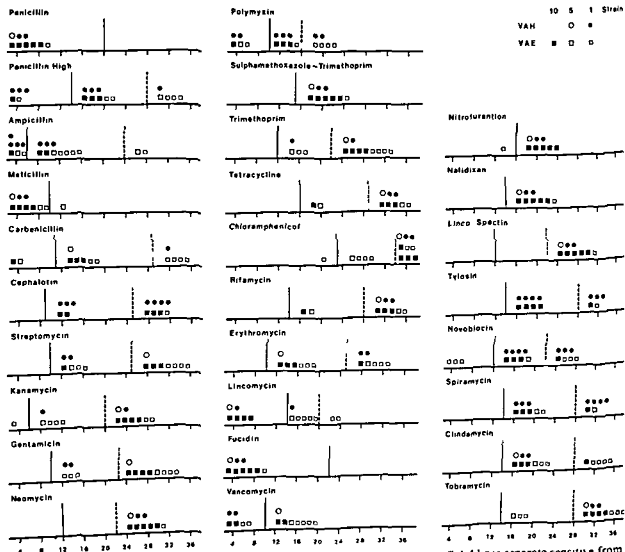


Fig 1 Inhibition zones for VAE and VAH strains with the employed antibiotics Solid lines separate sensitive from resistant strains Broken lines devide highly *from intermediately* sensitive strains

path microbiol scand Sect B 88 311-315 1980

AN *IN VITRO* EVALUATION OF TWO COMMERCIAL BLOOD CULTURE MEDIA – SUPPLEMENTED PEPTONE BROTH AND SUPPLEMENTED TRYPTONE SOYA BROTH

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Sandven, P & Høiby E A An *in vitro* evaluation of two commercial blood culture media – supplemented peptone broth and supplemented tryptone soya broth Acta path microbiol scand Sect B 88 311-315 1980

Two commercial blood culture media – supplemented peptone broth and supplemented tryptone soya broth – were compared by an *in vitro* method The two media were inoculated with microorganisms suspended in fresh human blood in an attempt to mimic the clinical situation The two media differed completely in their ability to support the growth of *Neisseria meningitidis* and *Neisseria gonorrhoeae* Macroscopic evaluation of the blood culture medium during the incubation period was difficult when using supplemented tryptone soya broth in contrast to supplemented peptone broth The former medium remained turbid whether microorganisms were growing or not No other important differences between the two media were detected Our findings indicate a need for both *in vitro* and *in vivo* studies to evaluate blood culture media and also a need for quality control of microbiological diagnostic devices before they are released for clinical use

Key words Blood culture media *Neisseria meningitidis*

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The detection of bacteraemia is one of the most ...portant tasks in clinical bacteriology A number ...clinical and *in vitro* studies have shown that the ...e of blood culture medium used influences the ...overy of microorganisms Ideally all such media ...ich are commercially available ought to be ...luated in clinical studies before being released for ...eral sale but because such *trials are time-...suming* and expensive this is not the case today ...alternative is *to evaluate blood culture* media by *...vitro* experiments It is however necessary to ...mic the *clinical situation* as closely as possible ...e have tried to do this by using fresh human *...ole blood* for the experiments The two blood ...ure devices compared in this study are suitable ... the cultivation of only two ml of blood and ...ould according to accepted recommendations (I ...only be used for pediatric blood cultures *In spite* ...these recommendations these blood culture ...ices are still commonly used in Norway today

MATERIALS AND METHODS

Blood culture media Vacutainer blood culture tubes with [illegible]

modified experimental medium containing 1 2% gelatin and lacking agar (STSBG) were obtained from Terumo Corporation Japan The ingredients of SPB and STSB are shown in Table 1 All tubes contained 18 ml of medium determined for an inoculum of approximately 2 ml of blood The concentration of sodium polyanethol sulfonate (SPS) in SPB was 0 025% and in STSB 0 035%

Microorganisms Recent clinical isolates obtained from the diagnostic microbiology laboratory of the insutute were used Strains isolated from blood cultures were omitted in order to avoid selection of strains already having grown in one of the blood culture media we were testing The clinical isolates used were 2 strains each of *Escherichia coli* *Proteus mirabilis* *Klebsiella* sp *Pseudomonas aeruginosa* *Haemophilus influenzae*

except one are sensitive to Trimethoprim Tetracycline Chloramphenicol Nitrofurantoin and Tobramycin These results are in agreement with investigations sporadically performed on isolated human pathogenic strains reviewed (1 15) However sulfa drugs were not tested in most reports

Cephalotin was 100% effective against VAH and 92% against VAE This was also found in two human pathogenic strains recently isolated in USA (14) while resistant strains were previously reported (10 12 13 15) Two tested strains of VAH were resistant to Clindamycin which is also recorded by *Schmidt et al* (14) but not encountered in other papers

About 70% of the strains were sensitive to Polymyxin (Colistin) It was previously reported to be effective with the exception of the recent two strains isolated (14) Although reports deal with high numbers of Ampicillin resistant strains (5 10 12 13 15 17) a moderate incidence of Ampicillin Vancomycin and Novobiocin resistant strains could be detected in the VAE and VAH strains

A high percentage of resistance was found to Meticillin Carbenicillin and Lincomycin while all strains show resistance to Penicillin and Fucidin Especially results of Carbenicillin and Ampicillin resistance are well documented by most authors (10 12 13 14 15 17)

In conclusion these findings show great correlation in sensitivity patterns among VAE and VAH strains and no variation in environmental strains isolated from different geographical areas The results could offer a basic information about the broad action of various antimicrobial agents on *V alginolyticus in vitro* and might serve as a guidance in therapy

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The technical assistance of Mrs *Kirsten Kaas* is acknowledged

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BLE 3 *Growth Pattern of Identical Inocula of* Pseudomonas aeruginosa Bacteroides fragilis N meningitidis *and* N gonorrhoeae *in the Blood Culture Media Tested*

croorganism	No of organisms in the inoculum	Blood culture medium	Growth[a]) by subculture in parallel blood culture tubes after	
			18 hours	42 hours
cudomonas	4	SPB	+++	+++
		STSB	---	+++
eud monas	4	SPB	+++	+++
		STSB	+++	+++
fragilis	6	SPB	--+	+++
		STSB	+++	+++
fragil s	12	SPB	+++	+++
		STSB	+++	+++
gonorrhoeae	12	SPB	+++	+++
		STSB	---	---
gonorrhoeae	2	SPB	---	-++
		STSB	---	---
		STSBG	---	---
mening i d s	14	SPB	+++	+++
		STSB	---	--
		STSBG	---	---
mening i d s	6	SPB	+++	+++
		STSB	---	---
		STSBG	+++	+++
meningit d s	210	SPB	++	++
		STSB	--	--
		STSBG	--	--
	36	SPB	-+	+
		STSB	--	-
		STSBG	--	-
men ngit d s	180	SPB	-+	+
		STSB	++	++
		STSBG	++	++
	18	SPB	++	++
		STSB	--	++
		STSBG	+	+
meningit d s	32	SPB	-++	-++
		STSB	--	--
		STSBG	---	---
	2	SPB	--+	-++
		STSB	---	--
		STSBG	--	---
men ngitidis	16	SPB	+++	[illegible]

TABLE 1 *Formulae of SPB and STSB Blood Culture Media*

Ingredient	SPB	STSB
	(grams per liter)	
Yeast extract	9 4	5 0
Tryptic digest of casein	4 7	
Enzymatic hydrolysate of meat	4 7	
Tryptone		17 0
Soya peptone		3 0
Meat extract		3 0
Liver digest		1 0
Gelatin	12 0	
Sodium bicarbonate	2 2	
Dextrose	2 5	
Glucose		2 5
Sodium chloride	4 0	5 0
K_2HPO_4		2 5
Adenine sulfate	0 01	
Magnesium sulfate	0 2	
Sodium polyanethol sulfonate	0 25	0 35
Cystein HCl	0 26	0 45
Sodium phosphate (dibasic)	0 14	
Para aminobenzoic acid	0 05	0 05
Proline	0 05	
Glutamic acid	0 05	
Nicotinamide adenine dinucleotide	0 0025	
Cocarboxylase	0 0003	
Vitamin B 12	0 0001	
Menadione sodium bisulfite	0 0002	
Hemin	0 005	0 005
Guanine HCl	0 0003	
Purified agar		0 01

Staphylococcus aureus Streptococcus pneumoniae Streptococcus pyogenes Streptococcus faecalis Streptococcus viridans Bacteroides fragilis ssp fragilis Candida albicans Neisseria gonorrhoeae and 6 strains of *Neisseria meningitidis*

Preparation of the inoculum Human blood was obtained by venipuncture immediately prior to use from two healthy donors (A H and P S) who had not received antibiotics during the previous months For each

lood

2 5

ately

mixed The microorganisms were grown on agar plates for 18–24 hours at 37 °C The anaerobic organisms were incubated in a Gas Pak® jar Organisms were carefully removed from the plates with a platinum loop and suspended in 0 9 % phosphate buffered saline (PBS) pH 7 4 The optical density of each suspension was measured and adjusted to give an optical density of 0 6 at 620 nm The suspension was afterwards serially diluted to give a suitable number of microorganisms per ml of PBS The size of the inoculum was chosen to mimic a low grade bacteraemia and at the same time to avoid such small numbers of microorganisms that an uneven distribution in the tubes resulted The exact number microorganisms used for inoculation was calculated determining the average colony forming units fro plating 0 1 ml samples on three chocolate agar plat Colonies were counted after 48 hours of incubation 37 °C

One ml of the suspension of microorganisms w inoculated into each of the portions of fresh blood Tl tubes containing the suspension of blood and microorg nisms were then thoroughly mixed One test organis was dealt with at a time in order to inoculate the bloc culture tubes as fast as possible The time used for ea organism never exceeded five minutes

Inoculation and evaluation of the blood culture med The rubber stopper of each blood culture tube w cleansed with 70% ethanol Two ml of the mixture blood and microorganisms was inoculated into the bloc culture tubes Three parallel tubes of each brand (SP and STSB) were inoculated in a random order Tw parallels were prepared for two of the meningococ strains Parallel tubes of STSBG were inoculated for o *N gonorrhoeae* strain and four *N meningitidis* strain The blood culture tubes were turned upside down to mi the contents and then vented by the use of venting uni of respective brand The media were incubated in air 37 °C At 18 h 42 h 66 h 90 h and after 7 and 14 day of incubation the tubes were inspected for evidence macroscopic growth and subcultured after thoroug mixing Six drops of the medium were removed throug the venting unit plated on chocolate agar and incubate at 37 °C with 5% CO_2 for 48 hours except for *B fragilis* which was incubated in a Gas Pak® jar

Agar diffusion test The sensitivity of 3 *N meningitidi* strains to SPB STSB STSBG and SPS was tested usin an agar diffusion test PDM – Antibiotic Sensitivit Medium AB Biodisk Stockholm (PDM medium) wa used for the tests The *N meningitidis* strains wer grown on chocolate agar for 18 hours and a suspensio containing approximately 10^4 colony forming units pr ml was made in 0 9% NaCl The surface of the mediun was seeded with 5 ml of this suspension giving a dens

TABLE 2 *No of Viable Microorganisms Used t Inoculate Each Blood Culture Tube*

	Inoculum[a]
E coli	4/2
Klebsiella sp	4/4
P mirabilis	8/8
H influenzae	4/2
Staph aureus	110/6
S pyogenes	6/4
S faecalis	12/22
S viridans	12/16
S pneumoniae	16/14
C albicans	56/60

a) Colony forming units inoculated per blood culture tube Two strains of each species were examined

both blood culture media contain SPS *Eng & and* (4) have shown that SPS may be harmful to *meningitidis* and *N gonorrhoeae* strains This ct was eliminated by adding 1 2% gelatin to the d culture media (3) Gelatin is incorporated in B but not in STSB It was therefore thought ly that the lack of gelatin in STSB was the n for the difference between SPB and STSB as ds the growth of *Neisseria* sp The experimen medium (STSBG) from Terumo (STSB con 1 2% gelatin and lacking agar) did not

w in SPB (Table 3) Other factors in addition to toxicity of SPS to *Neisseria* sp must therefore e into consideration to explain the poor growth *N meningitidis* and *N gonorrhoeae* in STSB

The diffusion test in agar indicates that SPB tains substances probably lacking in STSB that port the growth of *N meningitidis* In addition SB seems to contain substances that inhibit the wth of *N meningitidis* since a zone of reduced wth was produced around the well containing SB A part of this effect may be due to SPS The e of inhibited growth was however also noted en testing STSBG In this medium the toxic ct of SPS is probably neutralized by the gelatin tent of the medium It is therefore likely that er constituents in STSB may also contribute to toxic effect seen

The two media also differ as to the content of uble gases and pH (Table 4) The PO_2 is slightly her in SPB than in STSB (20 mm Hg and 12 mm respectively) The PCO_2 is also higher in SPB n in STSB (57 mm Hg and 6 mm Hg pectively)

Both SPB and STSB are rather complex media taining a number of undefined biological sub- ces such as soya peptone tryptone etc Evaluation of each component and combinations of them as to their effect on *N meningitidis* may therefore be complicated

The method of preparing bacterial suspensions in blood exposes the meningococci to SPS without gelatin (2) This factor operates equally on all parallels and cannot explain the differences seen

Although bacteraemias caused by *N meningitidis* are not rare in Norway it is doubtful if the important difference between the two blood culture media tested would have been detected in a clinical study This might indicate a need for both *in vitro* and *in vivo* studies to evaluate blood culture media

This study does reveal a need for thorough quality control of microbiological diagnostic devices before they are released for clinical use

We thank *Harald Goldstein* Institute of Economics University of Oslo for conducting the statistical analysis of the data presented in this study

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but not completely confluent growth The plates were allowed to dry for 30 minutes Wells (7 mm in diameter) were made in the medium and 0 1 ml of the substance to be tested was placed in the well The results were read after 18 hours of incubation in 5 % CO_2 at 37 °C

Analysis of soluble gases and pH Samples were taken by syringe and needle from two parallel blood culture tubes each of SPB and STSB and tested immediately for PCO_2 PO_2 and pH on a Corning pH Blood Gas Analyzer 165 before and immediately after inoculation with 2 ml fresh blood

RESULTS

The inocula used for the *E coli Klebsiella P mirabilis H influenzae Staph aureus S pyogenes S faecalis S viridans S pneumoniae* and *C albicans* strains are shown in Table 2 For these microorganisms no difference was observed between the two media tested The *H influenzae* and *S viridans* strains and one *C albicans* strain were detected on subculturing after 42 hours of incubation The other strains were detected after 18 hours of incubation The results obtained on subculturing for the other microorganisms used are shown in Table 3 For the whole study no blood culture negative at 42 hours showed growth on subculture later on

Small differences between the two blood culture media are noted for one of the *Pseudomonas* strains used and for one of the *Bacteroides* strains For the *Neisseria* strains however the differences between the two media are statistically highly significant ($p < 10^{-6}$) SPB supported the growth of all strains tested but only one of the eight strains was able to grow in STSB The experimental medium containing 1 2% gelatin and lacking agar STSBG were compared with the other two media (SPB and STSB) using 4 *N meningitidis* strains and 1 *N gonorrhoeae* strain (Table 3) Only for two strains was growth detected in STSBG

The agar diffusion test on PDM medium using 0 1 ml of 0 025% SPS or 0 035% SPS in the well yielded a zone of no growth around the well The diameter of the zone varied from strain to strain but all meningococcal strains investigated were inhibited by SPS Around the well containing SPB a zone of enhanced growth of *N meningitidis* was found in contrast to growth of smaller colonies on the rest of the agar surface A zone of markedly inhibited growth of *N meningitidis* was found around the wells containing STSB or STSBG The size of this zone was approximately equal to the zone of no growth found around the well containing 0 1 ml of 0 035% SPS The pH PCO_2 and PO_2 of SPB and STSB before and after the addition of fresh blood are shown in Table 4

TABLE 4 *Analysis of Soluble Gases and pH in SPB* [and] *STSB*

Blood culture medium	Before addition of blood			Immediately after addition of blood		
	pH	PO_2*	PCO_2*	pH	PO_2	PC[O_2]
SPB	7 1	20	57	7 2	34	3[illegible]
STSB	7 0	12	6	7 0	30	1[illegible]

* mm of Hg

The tubes inoculated with blood and microorganisms were compared with a negative control tu[be] containing only blood from the same donor [for] evidence of macroscopic growth For most of t[he] microorganisms used macroscopic growth w[as] easily detected in SPB This medium remained cle[ar] when negative on subculture Macroscopic signs [of] growth of *N meningitidis* and *N gonorrhoeae* we[re] usually detected 2 or more days after growth h[ad] been detected by subculturing When STSB w[as] used macroscopic evaluation was very difficult Th[e] medium remained turbid whether microorganism[s] were growing in the medium or not Macroscop[ic] growth was detected only when visible coloni[es] were present in the medium if hemolysis occurre[d] or if massive opacity resulted

DISCUSSION

The present study does of course suffer fro[m] limitations inherent when using an in vitro metho[d] The number of strains tested for each species are except for the meningococci too small to permit valid conclusion as to the growth supporting abilit[y] of the two blood culture media and this part of th[e] study was meant as a screening only The strain[s] used were freshly isolated strains from clinica[l] material It is however obvious that bacteria fron patients with septicaemia may be more difficult to grow than strains already having been isolated or bacteriological media Taking these limitations into consideration it is probable that only comparatively large differences between the two blood culture media tested can be detected by an in vitro method In spite of this we have been able to detect important differences between them Macroscopic evaluation of SPB was usually easy in contrast to STSB Using the latter is therefore likely to impose a lot of extra work on the bacteriological laboratory since repeated subculturing and microscopical examination will be necessary to ensure that there is no growth in the medi

RESISTANCE TYPES IN *ESCHERICHIA COLI*

II Transfer of Ampicillin Resistance

PER SØGAARD

Statens Seruminstitut, Regional Laboratory Odense University Hospital Odense Denmark

gaard P Resistance types in *Escherichia coli* II Transfer of ampicillin resistance Acta path icrobiol scand Sect B 88 317–322 1980

he ability to transfer resistance traits was investigated in 49 *E coli* strains isolated from clinical ecimens The strains were divided into three groups according to sensitivity to penicillin derivatives roup 1 contained 8 ampicillin-carbenicillin sensitive A s/Ca s) strains group 2 contained 16 npicillin resistent-carbenicillin sensitive (A r/Ca-s) strains and group 3 contained 25 ampicillin irbenicillin resistant strains In group 3 17 strains could transfer A resistance (range of transfer equency was $10^{0.0}$ to $10^{-7.5}$) The 16 strains in group 2 did not transfer A resistance more often than utants arose from the recipient $10^{-8.8}$) The mutants of the recipient selected on A plates were A r/ a s/cephalothin resistant exactly as the strains in group 2 The A resistance in group 3 was probably ased on plasmids and that of group 2 was based on mobilizable plasmids and/or chromosomal sistance The frequencies of transfer of sulphonamide tetracycline and streptomycin resistance of the rains in groups 1 and 2 that did transfer were in the same range as the frequencies of transfer in roup 3

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ansferable and non transferable antibiotic re ce in enteric bacteria was investigated by *Berg- et al* (1972) They found that ampicillin tance (A R) could be transferred in about 25% he A resistant (A r) strains Highly resistant rs could often transfer A R and mediated tance to very high concentrations of the biotic in the recipient

r strains of *E coli* can be divided into enicillin sensitive (Ca s) and Ca r strains a*rd* (1979) found the former group less stant to A and more resistant to cephalothin (Ce) n the latter one

Therefore an investigation was carried out to mine whether the frequency of transfer of A R s different in the two groups

MATERIALS AND METHODS

A Strains

The 49 clinical isolates were divided into the 3 groups shown in Table 1 The strains appear in the same order in Tables 2 3 and 4 in this paper as in *Søgaard* (1979)

B Media

Medium 1 Modified Conradi Drigalski plates con taining nalidixic acid (Nal) 25 µg/ml

The modified Conradi Drigalski plates have the following composition (*Gaarslev* 1980) demineralized H_2O 1 liter peptone (A/S Orthana DK 2770 Kastrup) [illegible]

thiosulphate 2 ml 50% bromthymol blue 10 ml 1% sodium dodecylbenzolsulfonate (Maranil pasta A 75

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Key words *Escherichia coli* resistance types transfer of ampicillin resistance

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Transferable and non transferable antibiotic resistance in enteric bacteria was investigated by *Berger et al* (1972) They found that ampicillin resistance (A R) could be transferred in about 25% of A resistant (A r) strains Highly resistant strains could often transfer A R and mediated resistance to very high concentrations of the antibiotic in the recipient

A r strains of *E coli* can be divided into carbenicillin sensitive (Ca-s) and Ca r strains *Søgaard* (1979) found the former group less resistant to A and more resistant to cephalothin (Ce) than the latter one

Therefore an investigation was carried out to determine whether the frequency of transfer of A R was different in the two groups

MATERIALS AND METHODS

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The modified Conradi Drigalski plates have the following composition (*Gaarslev* 1980) demineralized H_2O 1 liter peptone (A/S Orthana DK 2770 Kastrup) 10 g yeast extract (Oxoid L 21 Oxoid limited Basingstoke Hampshire Great Britain) 5 g NaCl 5 g Japan agar powder (Nordisk Droge DK 2100 København Ø) 11 g lactose 27 ml 33% glucose 1 2 ml 33% sodium thiosulphate 2 ml 50% bromthymol blue 10 ml 1% sodium dodecylbenzolsulfonate (Maranil pasta A 75

TABLE 1 *E coli Strains Used*

Strain	Phenotype	Source
Clinical isolates group 1 (n = 8)	A s/Ca s	*Søgaard* (1979)
Clinical isolates group 2 (n = 16)	A r/Ca s	*Søgaard*
Clinical isolates group 3 (n = 25)	A r/Ca r	*Søgaard*
K 12 W3132*nal*[a]	F⁻Met⁻ Nal r	*I Ørskov*
K 12 1100 *F Km*	F Km r	*K Nordstrom*

Abbreviations A = ampicillin Ca = carbenicillin Nal = nalidixic acid Km = kanamycin s = sensitive r = resista
Met = methionine a) = a naldixic acid resistant mutant of strain W3132 (*Søgaard* 1973)

Brøste P A/S DK 1415 København K) 1 ml 5% pH adjusted to 7 7–7 8

As appropriate the plates were supplemented by sulphonamide (Su) 100 µg/ml tetracycline (T) 20 µg/ml *streptomycin (S) 20* µg/ml chloramphenicol (C) 20µg/ml or A 10 15 20 or 25 µg/ml The plates with Su were peptone free

Medium 2 *The plasmid transconjugants were tested* on methionine (Met) free and Met containing plates of the following composition K_2HPO_4 7 g KH_2PO_4 3 g sodium citrate 3 H_2O 0 5 g $MgSO_4$ 7 H_2O 0 1 g $(NH_4)_2SO_4$ 1 g glucose 5 g agar 2 5 g H_2O 1000 ml The Met plates contained 10 µg Met/ml

Medium 3 Resistance plates (*Casals & Pedersen 1980 p 6–7*)

Medium 4 5% blood plates (*Lautrop et al* 1979)

Medium 5 Infusion broth

Medium 6 Water agar (2 5% Bacto agar in distilled H_2O)

Medium 7 Agar plates (concentrated ox broth with agar 1 7%)

All substrates were obtained from the substrate department Statens Seruminstitut Copenhagen

All cultures were done at 35 °C

C Direct Transfer Assay

The transfer was performed as described by *Bergfors et al* (1972) The donors were grown in medium 5 to a density of approx 10^8 CFU/0 1 ml (mean log CFU/0 1 ml 7 98 s d 0 48 range 6 0–8 7) The recipients were *grown in medium 5 with shaking to a density of approx* 10^8 CFU/0 1 ml (mean log CFU/0 1 ml 7 88 s d 0 45 range 6 8–8 5) Donor culture (0 1 ml) was mixed with 1 0 ml of recipient culture The mixture was incubated at 35 °C for 2 h before 10 ml *of medium 5 was added* After overnight incubation the broth was diluted 10^2 10^4 and 10^6 0 1 ml of undiluted and 0 1 ml of the 3 *dilutions were spread on appropriate plates of medium 1* Transfer of resistance to each antibiotic was tested on separate plates If any colonies appeared on the plates two were used for resistance determination on medium 3 with the agar diffusion technique (*Casals & Pedersen* 1980) to Su T S C Ce A Ca and Nal to confirm the transfer of resistance traits Material from the colonies was also tested on *Met free and Met-containing* medium 2 to ensure that the conjugants had the phenotype Met⁻ of the recipient All *the donors could grow on Met f* medium 2

From an overnight culture of donor and recipient ml was spread as control on the same plates as the mi culture

The number of CFU in the mixed culture determined both on *medium* 1 and *medium* 4 T *difference between the two counts was the number* donors in the mixed culture

D Mobilization Assay

This assay was done in two steps

Step 1 The *E coli* strain K 12 1100 *F Km* was us as donor of the F factor Nine clinical isolates of grou (*A r/Ca s*) were recipients The transfer was done described in C A r/Km r conjugants were isolated medium 1 with A 20 µg/ml and kanamycin 20 µg/ but without Nal

Step 2 The Km r clinical isolates from step 1 we used in a direct transfer with *E coli* K 12 W3132 *nal* recipient as described in C

E Resistance Test

This test was made as an agar diffusion test (*Casals Pedersen* 1980) using medium 3 and antibiotic di (Neosensitabs®) A strain with diameter of inhibit zone less than 23 mm was called resistant to t antibiotic in question

F Colicin Production Test

If the recipient (W3132) was eradicated from t mixed cultures of donor and recipient the donor w suspected of producing colicin This was shown follows A colony of the donor was grown on an ag plate (*medium 7*) *An overnight culture of the recipie* (W3132) was made 2 ml *of hot liquid medium 6* w mixed with 2 ml of recipient culture and poured over t agar plate with the donor colony The plate was th incubated overnight at 35 °C The formation of a cle zone without growth around the donor colony show the colicin production

G Materials

The following antibiotics were used in the plate Aminobenzylpenicillin D and carbenicillin (Fugacillin® Astra A/B Sodertalje Sweden Sulfadimidin Danmar

ugteterfo ening (DAK) DK 1611 København V
ᵣacyklin, DAK Dihydrostreptomycin sulfat, Nord sk
Dᵣgᵣ DK 2100 København Ø Kloramfen col DAK
ud ic ac d Sterl ng Winthrop A/S DK 2100 Kø
nha n Ø Kanamycinsulfat, Lundbeck DK 2500
lby

RESULTS

2 shows the frequency of transfer of A R In 3 (A r/Ca r) 17 of 23 strains were able to fer their A R usually with high frequency strain produced colicin (col) and one was

group 2 (A r/Ca s) 5 strains were omitted g to col formation or Nal R Among the

.E 2 *Frequency of Transfer of Amp c ll n Res s* *e among 41 Amp c ll n Res stant* E col *Stra ns*

ance	A r/Ca s (group 2) n 16	A r/Ca r (group 3) n 25
C		1 7
		5 7
		7 5
i		3 4
		2 7
	Nal r	
		col
:		
		−1 5
		−0 7
		−6 2
	7 9	−6 1
		−6 0
	col	
		Nal r
	col	0 0
		0 0
	Nal r	3 9
	Nal r	−0 3
		2 6
		−0 4
	−	−2 2

ency is expressed as log to the ratio between the her of A r rec p ents and the number of donors Su honamide T tetracycl ne S streptomyc n chloramphenicol O no res stance to Su T S or C no transfer observed col col cin produced by the e legend to Table I

remaining 11 strains only one (resistance type SuS) could apparently transfer its A R in the first attempt The transfer frequency of this strain was very small 10 7 9 (Su or S R was not transferred) The difference in frequency between the two groups is significant by Wilcoxon s test (p < 0 01) The experiment was repeated in the same manner for all 11 strains except the two strains that transferred SuTS and T R respectively (Table 3) One (resis tance type O) out of these 9 strains apparently transferred A R (frequency 10 7 4) The strain mentioned above did not transfer A R in this experiment

When 2×10^9 bacteria of the recipient W3132 were spread on medium 1 with A (20μg/ml) + Nal (25 μg/ml) 3 colonies of A r bacteria were observed (frequency $10^{-8\ 8}$) These three mutants were Ca s and Ce r as the strains in group 2 The frequency of S r mutants of the recipient was also 10 8 8 No Su T or C r mutants were observed Nal r mutants of the donors were observed with a frequency of 10 7 5

As mentioned by *Søgaard* (1979) 2 strains in group 3 had the same resistance profile as the bacteria in group 2 *i e* less resistant to Ca than to A and very resistant to Ce No A R transfer was observed with these 2 strains One could transfer Su R (frequency 4 3) and the other produced colicin (Table 3)

Table 3 shows the transfer of the resistance traits Su T S and C Su R was transferred from 2 (6) 1 (7) and 9 (16) strains in groups 1 2 and 3 respectively Figures in parentheses show the number of strains tested T R was transferred in 1 (2) 2 (3) and 5 (8) cases in groups 1 2 and 3 respectively The corresponding numbers for S R were 2 (6) 1 (7) and 8 (16) C R was transferred from 5 (8) strains in group 3

The transfer frequency of A R of each strain in group 3 was approx the same as the transfer frequencies of Su T S and C R (as applicable) corresponding to simultaneous transfer of all resistance traits (Table 2 and 3)

In group 2 none of the two strains that transferred SuTS and T R respectively transferred A R

The mobilization assay was done with the 9 group 2 strains mentioned in the second paragraph The results are shown in Table 4 A R could be transferred from 4 strains The highest frequency was $10^{-4\ 1}$ The bacteria of the observed colon es on the A plates were A r/Ca s/Ce r and had no other resistance traits except one which showed T R The transfer frequency of T in this mating was very high (10 1 0) Three out of the 5 strains with resistance types SuTS and SuS could transfer Su T and/or

TABLE 3 *Transfer of Resistance Traits (Ampicillin Excluded) According to Resistance Type among 33 E coli Stra*

Resistance *type*	A s/Ca s (group 1) *n* = 8	A r/Ca s (group 2) n – 8	A r/Ca r (group 3) n – 17
SuTSC			SuTSC (–0 3 –0 5 –0 6 –3 7)
			–
			SuTSC (–6 9 –6 3 –5 7 –6 3)
			SuTSC (–6 8 –7 3 –7 3 –7 4)
SuTS	Nal r	SuTS (–3 1 –2 7 –4 9)	SuTS (–3 6 –3 4 –5 3)
	SuS (–3 8 –4 0)	–	–
	Nal r	T (–1 3)	T (–2 5)
	T (–6 8)	Nal r	–
			col
SuSC			–
			SuSC (–1 5 –1 6 –1 6)
			SuSC (–0 7 –0 6 –0 5)
			–
SuS	–	–	–
	SuS (–3 9 –4 0)	–	SuS (–5 8 –5 5)
	–	–	Su (–4 3)
	–	–	SuS (–5 0 –5 0)

Abbreviations See legend to Table 2
Parentheses show the transfer frequency of corresponding resistance trait

S R None of the colonies formed showed A R 12 out of 22 colonies tested showed Km R

TABLE 4 *Transfer of Resistance Traits in Mobili ation Assay of 9 A r/Ca s* E coli *Strains*

Resistance type	
SuTS	SuTSA (–6 6 –1 0 –6 3 –6 8)
	Nal r
SuS	SuS (–6 4 –6 4)
	SuSA (–5 6 –5 7 –4 1)
	A (8 0)
	–
O	col
	col
	A (–7 9)
	Nal r
	Nal r
	–
	–
	–

– strain not tested
See legend to Table 3

DISCUSSION

The results suggest that the A R seen in group was based on plasmids while the A R in group were not transferred in the direct matings (Table 2 The other resistance traits could be transferred fron some of the strains in groups 1 and 2 (Table 3) Th transfer frequencies of these resistance traits i groups 1 and 2 were as high as the frequencies group 3

From Fig 3 of the paper by *Bergfors et al* (197 it can be concluded

a A r strains with high resistance can oft transfer their resistance and confer a high degr of resistance in the recipient
b A r strains with lower resistance cannot usual *transfer their resistance*

The results in this paper confirm these finding and show that *the group of A r/Ca s* E coli *strain comprises or at least belongs to the strains mentione above under b*

Group 3 (A r/Ca r) could transfer A R ver often and had a high degree of resistance Thi agrees well with production of class III β lactamases as these enzymes are plasmid mediated and have a high activity against A (*Richmond & Sykes* 1973) Plasmids with genes for class III β lactamases are known to be widespread

In the matings with donors of group 2 A r

es were found with a small frequency in two s. These colonies consisted of A r/Ca-s/Ce r ts. The overall frequency for A-R »transfer« oup 2 was $10^{-9.0}$ This frequency does not from the frequency of spontaneous A-r ts ($10^{-8.8}$) obtained in the control experiments hs) These mutants were A-r/Ca-s/Ce r, *i e* ame phenotype as the strains in group 2 fore it is a possibility that the group 2 strains roduced by mutation The mobilization assay ver showed that A R transfer from some of rains in group 2 could be established by means F factor (Table 4) The frequencies were small strain though showed transfer of A-R with a ency of $10^{-4.1}$ This differs from the frequency r mutants of the recipient ($10^{-8.8}$) It seems able that this strain had a mobilizable plasmid rring A- and Ce R

he A R in group 2 (A-r/Ca s) could be ined by a chromosomal penicillinase, a plas-mediated penicillinase and/or intrinsic resis-e *Burman et al* (1968) compared two isogenic ns of *E coli* K-12 one with a plasmid and one out. The former strain had a plasmid mediated tamase with higher activity against A than the mosomally mediated β lactamase of the latter in. 20µg/ml was the highest concentration mitting growth for the strain without a plasmid against 100 µg/ml A for the strain with a mid The mean IC_{50} with A of group 2 (A-r/s) in this investigation was 81 µg/ml and of p 3 (A-r/Ca r) ≥ 1112 µg/ml (*Søgaard* 1979) inoculum was much greater in the latter than in former investigation ($10^{4.7}$ vs $10^{1.0}$ CFU/ml) hromosomally mediated resistance to A invol-as the first step the *ampA* gene (*Eriksson Grenn-g* 1968) Higher resistance to A is obtained by addition of the *ampB* gene (*Nordstrom et al* 3) The sensitivity to Ca and Ce of *E coli* strains h these genes was not recorded The spontaneous mutants of the recipient and the group 2 strains this paper might belong to such strains

The strains in group 2 (A-r/Ca s) were very istant to Ce β lactamases of enzyme class I have gh activity against cephalospor n (Rich...

...smid mediated A R in group 2 deserves some nsideration The »plasmids« could not be trans-red in the direct assay but the A R was ectable in some of the strains *Bobrowski et al* (1976) reported the finding of a plasmid mediated β ctamase indistinguishable from the chromosomal lactamase of *E coli* They mentioned the hypothe-s that their plasmid originated from an *E coli* chromosome This plasmid in an *E coli* K-12 host gave resistance to A 32 µg/ml, Ca 8 µg/ml and Ce 125 µg/ml (inoculum 10^{2} CFU) That was the same profile as the group 2 strains had in this investigation, and the resistance levels are about the same (A 81 µg/ml, Ca 15 µg/ml, Ce ≥ 421 µg/ml, inoculum $10^{4.7}$ CFU/ml) (*Søgaard* 1979) Thus it is possible that some of the group 2 bacteria harbour a mobilizable plasmid like that of *Bobrowski et al* (1976)

Another explanation of the A-R observed in group 2 could be intrinsic resistance In that case the outer membrane should be more permeable to Ca than to A and Ce Plasmid free *Pseudomonas aeruginosa* strains have the same profile Some mutants of these strains have almost the same low MIC to A, Ca and cephalosporin (*Richmond & Sykes* 1973, Table 21) The resistance of the former strains is interpreted as intrinsic resistance, while the mutants have lost theirs

There is also the possibility that the A-R of group 2 strains is based both on chromosomally-mediated penicillinase and intrinsic resistance as described by *Boman et al* (1974)

The nature of the A-R in group 2 (A-r/Ca s) can be further clarified by β-lactamase investigations

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I am indebted to Miss *L -L B Jørgensen* for technical assistance

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URIFICATION AND CHARACTERIZATION OF ALEUTIAN DISEASE VIRUS

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asted B Purification and characterization of aleutian disease virus Acta path microbiol scand Sect 88 323-3?8 1980

irus was isolated from infected mink organs by a combination of tissue homogenization fluorocarbon traction and ultracentrifugation The final preparation was analysed by crossed immunoelectrophore s and electronmicroscopy Virions had a capsid diameter of 22 nm Preparative agarose ectrophoresis separated virions from contaminating ferritin Crossed immunoelectrophoresis of virus ve a single precipitate with sera from infected mink Crossed immunoelectrophoretic analysis with termediate gels showed that a part of the virus preparation was complexed with antibody Serum om a certain mink was found to contain precipitating antibody to (poly)nucleotid Virus and virus tibody complexes were found to focus at pH 4 0-4 4 in isoelectric focusing In SDS polyacrylamide el-electrophoresis the main virus protein was found to have a molecular weight of 69000 This study ves further support to the classification of aleutian disease virus as a parvovirus

ey words Aleutian disease virus Plasmacytosis Parvo viridae

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eutian disease of mink (plasmacytosis) is d by a persistent virus infection which gives a ed polyclonal hypergammaglobulinemia plexes of virus antigen antibody and comple t have been found in connection with glomeru tritis and vasculitis in the terminal stage of se (16 17)

lost of the published physical-chemical parame of aleutian disease (ADV) indicate that the virus parvovirus (13 16) These studies include the and density of the particles (7) and the presence irus-induced antigen(s) in the nucleus of infected s (11) However the only two existing reports on peptide analyses of ADV agree about the ence of three low molecular weight polypeptides ich are picorna virus like (15 20) and not racteristic for parvo-virus (19) Because of the ...ty that some of the chemicals used in the ndard purification procedure of ADV might ...ce breakdown products of the virus polypepti des we have chosen preparative electrophoresis as an alternative final purification method instead of density gradient ultracentrifugation which normally has been used (15 20) The intention of our studies has been to study the nature of the virus antigens by physical-chemical analyses and to characterize mink antibodies induced by the virus infection and thereby give an elucidation of the problem A preliminary report of the results has been given elsewhere (1)

MATERIAL AND METHODS

Infection of Animals

Sera from healthy mink were tested in countercurrent electrophoresis (8) and found to give a negative reaction against ADV antigen These mink were then injected with organ material isolated from infected mink and killed on day 10 after injection The exact procedure has been described by *Brummerstedt* (5)

Production of Virus Antigen

Virus was partially purified by tissue homogenization in phosphate buffered saline (50 mM phosphate, 100 mM NaCl, pH 7 0) freezing and thawing the homogenate 4 times and fluorocarbon extraction (2 weight parts of homogenate mixed with 1 weight part of fluorocarbon) as described partly by *Ingram & Cho* (13) The supernatant was collected and the tissue phase was re-extracted with fluorocarbon The combined supernatants were centrifuged at 7000 × *g* for 45 min to remove remaining solids and the supernatant was ultracentrifuged at 156000 × g for 2 h at 10 °C The pellet was re-extracted with fluorocarbon (The step is also referred to as »activation« of virus, i e liberation of immunogenic determinants on virus)

Preparation of Mink Immunoglobulin

The virus antigen preparation was immobilized by coupling to N-hydroxysuccinimide activated Sepharose 4B as described by *Cuatrecasas* (9) 1 ml of this matrix was packed into a column and 1 ml of a pool of sera from 20 infected mink was passed through the column The column was washed with 10 mM phosphate, 150 mM NaCl buffer pH 7 0 to A_{280} less than 0 01 and eluted with 0 2 M glycine buffer pH 3 0 The pH was finally adjusted to 7 0 with NaOH The eluted protein was analysed by crossed immunoelectrophoresis (2) and sodium dodecyl sulphate polyacrylamide gelelectrophoresis (14) and found to be immunoglobulin

Production of Rabbit Antisera

Rabbits were immunized subscapularly with 100 µg of either the virus-antigen preparation or mink immunoglobulin preparation in Freund's incomplete adjuvant for antiserum preparation

Crossed Immunoelectrophoresis

Crossed immunoelectrophoresis with and without the use of intermediate gels was carried out as described by *Axelsen et al* (2)

In some experiments Concanacalin A was used in the first dimension gel in a concentration of 100 µg per ml of agarose for glycoprotein identification (6)

Preparative Agarose Electrophoresis

Preparative agarose electrophoresis was carried out in 1 % agarose blocks of 120 × 100 × 5 mm in 20 mM barbital buffer pH 8 6 Electrophoresis time was about 2 h at 50 mA After electrophoresis the agarose block was cut into 20 pieces (120 × 5 × 5 mm) and a small part of each piece (5 × 5 × 5 mm) was laid directly on an agarose gel containing serum from an infected mink or rabbit antiserum to the virus-antigen preparation (rocket electrophoresis see ref 2) The agarose pieces positive for virus and ferritin (i e gave a precipitate in rocket electrophoresis) were centrifuged at 40000 × *g* in 30 min at 4 °C, and the supernatant was collected

For isolation of the (poly)nucleotide present in the virus antigen preparation, preparative agarose electro phoresis with continuous elution was carried out (Savant preparative electrophoresis apparatus model PAG 15) The same buffer system as above was employed

Isoelectric Focusing in Agarose Gels

Isoelectric focusing in agarose gels was carried o described by *Rosen et al* (18)

125-I Labelling

125-I labelling was carried out by the iodogen me (10) the chloramine method (12) or by the Bol Hunter method (4)

SDS-Polyacrylamidegel-Electrophoresis

SDS-polyacrylamidegel-electrophoresis was car out in slab gels as described by *Laemmli* (14) Sam were heated to 100 °C 1–2 min in sample buffer be electrophoresis

Electronmicroscopy

Electronmicroscopy was performed by *B Bloch* at department using uranyl acetate stain The method has previously been described (5)

RESULTS

Purification of Aleutian Disease Virus

Crossed immunoelectrophoresis of virus anti prepared by fluorocarbon extraction and differen

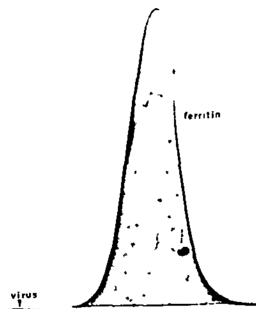
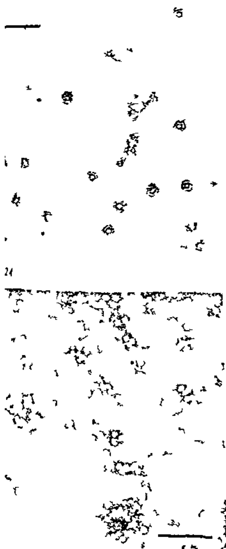


Fig 1 Crossed immunoelectrophoresis of 10 µl of viru antigen preparation with 8 3 µl rabbit antiserum to t antigen preparation per cm² of second dimensic agarosegel



1 Electronm c oscopy of virus (F g 2A) and ferr tin
: 2B) p eparations sola ed by preparative agarose
ctropho esis Magn f ca on 140000 × The bar repre
s 100 nm

trifugation (see Material and Methods) is shown
Fig 1 us ng a rabb t antiserum to the same
paration. Ferritin was the dom nating prote n as
viously shown by electronm croscopy (5) There
re at least 100 times more ferrit n molecules than
us particles

Ferritin and virus were separated by preparative
rose electrophoresis The purity of the isolated
ritin and virus contain ng fract ons was estimated
electronmicroscopy as shown in Fig 2 No
a structures were found in the virus prepara
n and vice versa

Analysis of Virus by Crossed Immunoelectrophoresis

The virus preparation was analysed by crossed immunoelectrophoresis against both a rabbit antiserum to the virus antigen preparation (antiserum against both ferritin and virus) and sera from infected mink (antiserum to virus) Only a single precip tation arch was found in a position corresponding to the reaction between virus and antibody (Fig 3)

When con A was incorporated into the first dimension agarose gel of a crossed immunoelectrophoresis no inhibition of electrophoretic migration was observed for virus This indicates that no con A binding sugar residues are coupled to the virus capsid polypeptides

Virus was radiolabelled with 125 I by three different iodination methods No d fference was found in iodination capacity of the different methods and generally only small amounts of iodine could be incorporated into the virus particles

When radiolabelled virus was analysed by crossed immunoelectrophoresis exactly the same precipitation arch was found as the one shown in Figs 1 and 3 Autorad ography of the electrophoresis is shown in Fig 4 Only one precipitation arch was found the one corresponding to the virus antibody reaction The form of this precipitation arch indicated that it consisted of a relatively strong symmetrical precipitation zone and a trail zone in the d rection of the application well This precipitate was analysed further in crossed immunoelectrophoresis with intermediate gels containing a rabbit antiserum to mink immunoglobulin(s) as shown in Fig 5 It can be seen that the rabb t antibod es in the



Fig 3 Crossed mmunoelectrophores s of 10 μl of v rus preparat on with 0 033 μl serum from an nfected m nk per cm² of second d mens on agarose gel

Fig 4 Autoradiography of virus antibody immune precipitate from crossed immunoelectrophoresis The arrow indicates the application slit

intermediate gel reacted only with the »trail zone« while the serum from the infected mink reacted mainly with the symmetrical (pure) virus component

Estimation of the Isoelectric Point of Virus

Isoelectric focusing in agarose gels of virus followed by identification of virus by a second dimension electrophoresis of the focusing gel into an agarose gel containing antiserum to virus elucidated that virus (and virus antibody complexes) focused at pH 4 0–4 4 Three precipitation arches were seen by this technique as shown in Fig 6

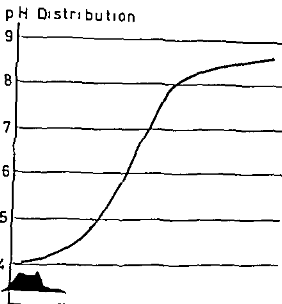


Fig 6 pH distribution in an isoelectric focusing agarose gel After focusing the gel containing 50 µl of virus was overlaid on a second dimension agarose gel containing 0 033 µl serum from an infected mink per cm² of agarose gel for virus identification

Demonstration of Free (Poly)Nucleotid in the Virus Antigen Preparations

A very fast moving molecule was found in certain virus antigen preparations when they were analysed in agarose electrophoresis The molecules migrated to more than double the distance of the virus and the virus is in itself a fast moving particle This fast moving molecule was isolated by preparative agarose electrophoresis An absorption spectrum was obtained and the isolated molecule had a maximum absorbance at 260 nm The

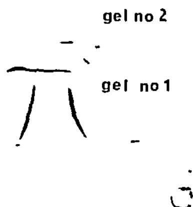


Fig 5 Crossed immunoelectrophoresis of 10 µl of virus preparation with 10 µl rabbit antiserum against mink immunoglobulin(s) per cm² of intermediate second dimension gel (gel no 1) and 0 03 µl serum from an infected mink per cm² of second dimension gel (gel no 2)

Fig 7 Crossed immunoelectrophoresis of 10 µl of virus antigen preparation with 0 033 µl serum from mink no 24 per cm² of second dimension gel The arrowhead indicates the application slit

cule could be stained with toluidine blue and in se electrophoresis it migrated to exactly the distance as a DNA preparation prepared from ted mink organs When serum from a certain k (No 24) was incorporated into a second nsion agarose gel precipitates were formed st both the virus and the new molecule as n in Fig 7

racteri ation of Virus Polypeptides

he virus preparation isolated from the prepara agarose gel electrophoresis was analysed by polyacrylamidegel electrophoresis with and out 2 mercaptoethanol as seen in Fig 8

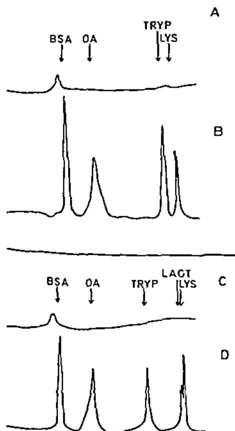


Fig 8 Densitometric scanning of an 11% SDS poly acrylamide gel of A 75 μl of virus preparation without 2 mercaptoethanol (2 ME) treatment
B 4 marker proteins 5 μg of each (BSA – Bovine Serum Albumin of M_r 66000 OA Ov Albumin of M_r 45000 TRYP Trypsinogen with M_r 24000 and LYS Lysozyme with M_r 14300) without 2 ME treatment
C 75 μl of virus preparation with 2 ME treatment
D 5 marker proteins 5 μg of each (the same 4 as in B + LACT beta lactoglobulin with M_r 18400) with 2 ME treatment

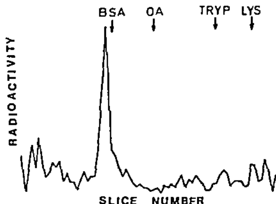


Fig 9 Radioactive distribution in an 11% SDS polyacrylamide gel of a 125 I labelled virus preparation

A single polypeptide with M_r 69000 was found In one similar experiment a faint second band with M_r 59000 was also seen 125 I labelled pure virus was also analysed by SDS polyacrylamide gel electrophoresis The radioactive distribution in such a gel is shown in Fig 9 and it confirms the presence of a 69000 molecular weight band as did radioautography

DISCUSSION

This study introduces a new electrophoretic method for virus purification Normally ultracentrifugation in gradients has been used for the final virus purification step (7 15 20)

Virus was found to be partly covered by antibody giving a trail zone in crossed immunoelectrophoresis

Virus (and virus antibody complexes) were found to focus at pH 4 0–4 4 in isoelectric focusing gels It should be pointed out that there is an uncertaincy in estmating isoelectric points at the end of a pH gradient as the one in Fig 6 However the pI values of 4 0–4 4 are consistent with the electrophoretic migration seen in normal agarose electrophoresis where virus migrated in front of human serum albumin used as a marker protein (pI of human serum albumin = 4 7) Because virus migration was not found to be inhibited by Con A incorporated into the agarose gel during electrophoresis it is likely that virus capsid proteins are not glycosylated Acid sugar residues coupled to virus proteins cannot therefore be responsible for the low isoelectric focusing point of virus

Three precipitation arches were found when the isoelectric focused virus and virus antibody complexes were analysed by rocket electrophoresis It is likely that the most acid particle (electrofocusing at

pH 4 0) is virus itself and the two other components are virus complexed with different ratios of antibody

Free (poly)nucleotide was found in some virus antigen preparations It is quite possible that the fluorocarbon extraction destroys some virus particles causing the liberation of virus (poly)nucleotide The ultracentrifugation procedure used for the virus antigen preparation will pellet some of this (poly)nucleotide

It was found that serum from a certain mink had antibody to this (poly)nucleotide The production of such antibodies in infected mink has been reported before (3 21) It is however uncertain how frequently sera from ADV infected and non ADV infected mink do contain such antibodies The latter group of sera will most likely give false positive reactions in countercurrent electrophoresis for the diagnosis of ADV infections (8) This question is under study in our laboratory

When various virus preparations were analysed by SDS polyacrylamide gel electrophoresis the main polypeptide was found to have a M_r of 69000 Occasionally a faint second band with M_r of 59000 was seen When the same analyses were performed with 125 I labelled virus the presence of the M_r 69000 molecular weight component was confirmed The 69000 and 59000 polypeptides found in this study of ADV are comparable to the polypeptides of the parvo viridae (19) The apparent inconsistency of the finding of these polypeptides with regard to the three low molecular weight polypeptides published by others (15 20) cannot at present by explained but it may be suggested that the introduction of preparative agarose electrophoresis as the final purification method instead of gradient ultracentrifugation gave parvovirus related polypeptides when purified virus was analysed in SDS polyacrylamide gel electrophoresis

The technical assistance of Miss *Lene Raabi* and Miss *Uli Jorgensen* is acknowledged The study was supported by a grant from the *Danish Fur Breeders Association*

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CHARACTERISTICS OF ANTISERA AGAINST PERIODATE-RESISTANT MEMBRANE ANTIGENS FROM *NEISSERIA GONORRHOEAE*

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Roe S F Eggset G Iversen O J & Mæland J A Characteristics of antisera against periodate resistant membrane antigens from *Neisseria gonorrhoeae* Acta path microbiol scand Sect B 88 329–334 1980

Crude outer membrane (OM) was prepared by extraction of bacteria of the *Neisseria gonorrhoeae* strains 8551 V and VII with an EDTA-containing buffer The preparations contained the lipopolysaccharide (LPS) and at least 10 proteins as shown by SDS-polyacrylamide gel electrophoresis Immunization of rabbits with untreated OM resulted in production of antibodies against several antigens including LPS Antisera raised against periodate treated OM did not contain antibodies against LPS These latter antisera agglutinated heat treated (100 °C 60 min) gonoccal cells by means of antibodies to one or more common agglutinogens and against a strain specific agglutinogen that was susceptible to digestion with proteolytic enzymes Both slide agglutination and a plate agglutination test could be used to detect antibodies against these agglutinogens

Key words *Neisseria gonorrhoeae* membrane antigens periodate resistance

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Gonococci are heterogeneous with regard to …genic specificity of some of the surface structu…s of the bacterial cells These structures include the …i of gonococci (3 15) and components of the …ter membrane (OM) such as the lipopolysaccha…de (LPS) and OM proteins (4 8 9 14) This …owledge has been attained by the use of different …boratory techniques some of which may be too …borious for routine serotyping of the bacterium …ently Sandstrom & Danielsson (17) reported …that when antisera to whole cells of gonococci were …ross absorbed and used in a co-agglutination test …eat-treated gonococcal cells could be classified into …ree antigenic classes each with a number of sub-…groups

Results reported from this laboratory have shown that both OM proteins and LPS function as …agglutinogens in heated gonococci (1) Hence serotyping of the bacteria on the basis of protein agglutinogens may be interfered with by anti LPS antibodies and vice versa

This report describes the results of experiments designed to raise antisera that contained antibodies to protein agglutinogens of the *Neisseria gonorrhoeae* strains 8551 V and VII but not antibodies to LPS Rabbits were immunized with OM oxidized with periodate to destroy LPS and the antisera obtained were compared with sera from rabbits immunized with untreated OM

MATERIAL AND METHODS

Bacterial Strains and Culture

The *N gonorrhoeae* strains 8551 V and VII colony types T3 and T4 were cultured and harvested as previously (1) except that the culture medium used (18) was supplemented with the following antibiotics Linco-

mycin chloride (1 μg/ml), Amphothericin B (1 μg/ml), Trimetoprim lactate (6 5 μg/ml), Colistin sodium methanosulphonate (6 5 μg/ml)

Preparation of Antigens

Mansheim & Kasper (10) The final preparations were suspended in distilled water, lyophilized, or kept frozen (8 mg/ml)

Lipopolysaccharide (LPS) LPS was prepared by extraction of whole gonococcal cells with phenol-water, and purified as reported earlier (12) To make it ready for the sensitization of sheep erythrocytes, lyophilized LPS was treated with NaOH and digested with pronase as reported (14)

Antigenic determinant b A crude preparation was prepared by the extraction of whole gonococcal cells with alkali (11) The preparation was treated with periodate to destroy LPS and was then used for the sensitization of sheep erythrocytes with the protein determinant *b* as described previously (12)

Serological Methods

Bacterial agglutination The titre of antiserum against heat-treated gonococci (100 °C 60 min) was determined using a glass plate agglutination test as reported (1) Slide agglutination testing was performed by the mixing of one drop of antiserum diluted in phosphate buffered saline pH 7 2 (PBS) with one drop of the suspension of heated gonococci standardized to a turbidity of a McFarland No 5 standard The slide was agitated by hand for up to 150 s and the agglutination read in oblique light was graded as follows agglutination appearing within 30 s (3 +) agglutination appearing from 30 to 90 s (2 +) agglutination appearing from 90 to 150 s (1 +) no agglutination after 150 s (–)

Indirect haemagglutination Sheep erythrocytes were sensitized with LPS or the *b* determinant of gonococci as described earlier (12) The assay was performed using a microtitre equipment and the titres of antiserum determined as described (6)

Immunodiffusion Double diffusion slides were prepared and the test was performed as described previously (2) The wells were filled with undiluted antiserum or with OM (4 mg/ml) dissolved in an endotoxin-disaggregating buffer (10) that contained Na-deoxycholate (NaD buffer) The gels were stained for proteins with Coomassie brilliant blue (21)

Other Methods

Immunization Rabbits were immunized with untreated or periodate-oxidized OM preparations suspended in 0 85 per cent NaCl The animals received 0 5 mg of the preparations intramuscularly twice each week for a period of three weeks and then 1 mg doses twice in the sixth and twice in the seventh week of the immunization period The animals were bled 7 days after the last injection The antisera obtained, anti OM from animals receiving untreated OM, and anti-pOM from those injected with periodate-treated OM, were stored at –20 °C, and heated at 56 °C for 30 min before testing

Absorption of antiserum Absorption with untreated or heat treated gonococci was performed as previously described (1) For the absorption, 0 2 g of wet bacteria was used per 0 1 ml of undiluted antiserum This same amount of antiserum was absorbed with 4 mg of the OM preparation with incubation and centrifugation as reported (1)

of the OM preparation in 0 7 ml volume of the same buffer was applied The gradient was centrifuged (Beckman Model L5 – 65) at 105000 × *g* for 18 h Fractions of 0 6 ml each were collected by a density gradient fractionator (Isco, Model 640) Then 0 1 ml of each fraction was mixed with 0 9 ml of 0 1N NaOH and the optical density was measured at 280 nm

Electron microscopy OM in distilled water (0 25 mg/ml) was prepared and stained with 2 per cent (w/v) sodium phosphotungstate, pH 6 6 for 30 s (5) The preparations were examined by electron microscopy (Jeol JEM-100 CX)

SDS-polyacrylamide gel electrophoresis Electrophoresis was performed essentially as described by *Weber & Osborn* (20) using the protein standards buffers and staining methods reported earlier (6)

Periodate oxidation The OM preparation was suspended in 0 5 per cent Na *meta*-periodate (Merck) in distilled water (1 mg/ml) kept at 4 °C for 20 h, dialyzed against water for 24 h and then against saline The preparations were kept frozen and were thawed immediately before being used for immunization

Enzyme digestion All the enzyme preparations were manufactured by Calbiochem Digestion with trypsin or pronase was performed using a 0 05M tris HCL buffer pH 7 5 with pepsin using a 0 05M citrate phosphate buffer pH 2 6 and with papain using a 0 1M phosphate buffer pH 7 4 with the addition of 0 01M cysteine and 0 002M EDTA The digestion was performed at 37 °C for 4 h with a substrate to enzyme ratio of 16 1 (w/w) The enzyme activity was destroyed by heating the preparation at 100 °C for 5 min The mixtures were then dialyzed against water lyophilized and used for the absorption of antiserum as described above An undigested control preparation was included for each of the enzymes

RESULTS

Electron microscopy of the OM preparations showed material consisting of aggregates of membraneous structures of variable shape, most of them bounded by a dense layer (Fig 1) On sucrose gradient centrifugation a single band between 1 18 and 1 23 g/ml of sucrose was observed (Fig 2) SDS-polyacrylamide gel electrophoresis showed that each preparation contained at least *10 different* proteins, some of which were present in very low



Fig 1 Electron micrograph of an outer membrane preparation from the *N gonorrhoeae* strain VIII negatively stained with sodium phosphotungstate pH (×90 000)

preparations (Fig 3) The preparations contained a dominating protein with a molecular weight of 39000 daltons for each of the strains 8551 and VII and 38000 daltons for strain V Staining for carbohydrates with Alcian blue showed a fast moving line in all three preparations with a mobility corresponding to that of cytochrome C (12500 daltons)

Double diffusion in agar gel analysis of the OM preparations against the corresponding anti OM or anti pOM sera showed similar patterns of precipitation lines with all three strains exemplified in Fig 4 Both antisera gave rise to a precipitation line close to the antigen basin and one line midway between the antigen and serum basins The anti OM but not the anti pOM serum also showed a line close to the serum basin This pattern of precipitation lines was seen when OM was dissolved in the NaD buffer No line was observed when the preparations were suspended in PBS and tested

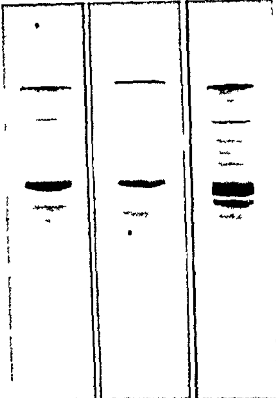


Fig 3 SDS polyacrylamide gel electrophoresis of the outer membrane from the *N gonorrhoeae* strains 8551 (left) V (middle) and VII (right)

Indirect Haemagglutination Testing

The anti OM sera contained antibodies both against LPS and the protein determinant *b* of the corresponding strains (Table 1) Antibody activity against determinant *b* was also shown by the anti pOM sera but they showed no antibody activity against LPS

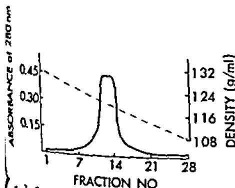


Fig 2 Sucrose gradient centrifugation of the outer membrane from the *N gonorrhoeae* strain VII Solid line indicates absorption at 280 nm and broken line density of sucrose



Fig 4 Double diffusion in agar gel with anti-OM 8551 (lower left) and anti pOM 8551 serum (lower right) against outer membrane from the *N gonorrheae* strain 8551 (upper well)

mycin chloride (1 µg/ml), Amphothericin B (1 µg/ml), Trimetoprim lactate (6 5 µg/ml), Colistin sodium methanosulphonate (6 5 µg/ml)

Preparation of Antigens

Outer membrane Extraction of whole gonococcal cells with an EDTA containing buffer and purification of the OM by centrifugation were performed as described by *Mansheim & Kasper* (10) The final preparations were suspended in distilled water, lyophilized, or kept frozen (8 mg/ml)

Lipopolysaccharide (LPS) LPS was prepared by extraction of whole gonococcal cells with phenol-water, and purified as reported earlier (12) To make it ready for the sensitization of sheep erythrocytes, lyophilized LPS was treated with NaOH and digested with pronase as reported (14)

Antigenic determinant b A crude preparation was prepared by the extraction of whole gonococcal cells with alkali (11) The preparation was treated with periodate to destroy LPS and was then used for the sensitization of sheep erythrocytes with the protein determinant *b* as described previously (12)

Serological Methods

Bacterial agglutination The titre of antiserum against heat treated gonococci (100 °C, 60 min) was determined using a glass plate agglutination test as reported (1) Slide agglutination testing was performed by the mixing of one drop of antiserum diluted in phosphate buffered saline pH 7 2 (PBS) with one drop of the suspension of heated gonococci standardized to a turbidity of a McFarland No 5 standard The slide was agitated by hand for up to 150 ... was graded ... 30 s (3+) ... 0 s (2+) ... s (1+) no agglutination after 150 s (−)

Indirect haemagglutination Sheep erythrocytes were sensitized with LPS or the *b* determinant of gonococci as described earlier (12) The assay was performed using a microtitre equipment and the titres of antiserum determined as described (6)

Immunodiffusion Double diffusion slides were prepared and the test was performed as described previously (2) The wells were filled with undiluted antiserum or with OM (4 mg/ml) dissolved in an endotoxin disaggregating buffer (10) that contained Na-deoxycholate (NaD buffer) The gels were stained for proteins with Coomassie brilliant blue (21)

Other Methods

Immunization Rabbits were immunized with untreated or periodate oxidized OM preparations suspended in 0 85 per cent NaCl The animals received 0 5 mg of the preparations intramuscularly twice each week for a period of three weeks and then 1 mg doses twice in the sixth and twice in the seventh week of the immunization period The animals were bled 7 days after the last injection The antisera obtained anti-OM from animals receiving untreated OM and anti pOM from those injected with periodate-treated OM were stored at − °C, and heated at 56 °C for 30 min before testing

Absorption of antiserum Absorption with untreated heat treated gonococci was performed as previously described (1) For the absorption 0 2 g of wet bacteria was used per 0 1 ml of undiluted antiserum This same amount of antiserum was absorbed with 4 mg of the OM preparation with incubation and centrifugation as reported (1)

Sucrose gradient centrifugation A linear gradient of sucrose, from 20 to 65 per cent (w/w) in a total volume of 16 ml was prepared in the EDTA buffer (10) and 7 of the OM preparation in 0 7 ml volume of the same buffer was applied The gradient was centrifuged (Beckman Model L5 − 65) at 105000 × *g* for 18 h Fractions of 0 6 ml each were collected by a density gradient fractionator (Isco, Model 640) Then 0 1 ml of each fraction was mixed with 0 9 ml of 0 1N NaOH and the optical density was measured at 280 nm

Electron microscopy OM in distilled water (0 25 mg/ml) was prepared and stained with 2 per cent (w/v) sodium phosphotungstate pH 6 6 for 30 s (5) The preparations were examined by electron microscope (Jeol JEM-100 CX)

SDS-polyacrylamide gel electrophoresis Electrophoresis was performed essentially as described by *Weber & Osborn* (20), using the protein standards buffers and staining methods reported earlier (6)

Periodate oxidation The OM preparation was suspended in 0 5 per cent Na-*meta*-periodate (Merck) in distilled water (1 mg/ml) kept at 4 °C for 20 h dialyzed against water for 24 h, and then against saline The preparations were kept frozen and were thawed immediately before being used for immunization

Enzyme digestion All the enzyme preparations were manufactured by Calbiochem Digestion with trypsin or pronase was performed using a 0 05M tris HCL buffer pH 7 5 with pepsin using a 0 05M citrate phosphate buffer pH 2 6 and with papain using a 0 1M phosphate buffer pH 7 4 with the addition of 0 01M cysteine and 0 002M EDTA The digestion was performed at 37 °C for 4 h with a substrate to enzyme ratio of 16 1 (w/w) The enzyme activity was destroyed by heating the preparation at 100 °C for 5 min The mixtures were then dialyzed against water, lyophilized and used for the absorption of antiserum as described above An undigested control preparation was included for each of the enzymes

RESULTS

Electron microscopy of the OM preparations showed material consisting of aggregates of membraneous structures of variable shape, most of them bounded by a dense layer (Fig 1) On sucrose gradient centrifugation a single band between 1 18 and 1 23 g/ml of sucrose was observed (Fig 2) ... phoresis showed ... proteins, some of ...



Fig 1 Electron micrograph of an outer membrane preparation from the *N gonorrhoeae* strain VIII negatively stained with sodium phosphotungstate pH 6 (×90 000)

concentrations (Fig 3) The preparations contained a dominating protein with a molecular weight of 36000 daltons for each of the strains 8551 and VII and 38000 daltons for strain V Staining for carbohydrates with Alcian blue showed a fast moving line in all three preparations with a mobility corresponding to that of cytochrome C (12500 daltons)

Double diffusion in agar gel analysis of the OM preparations against the corresponding anti-OM or anti-pOM sera showed similar patterns of precipitation lines with all three strains exemplified in Fig 4 Both antisera gave rise to a precipitation line close to the antigen basin and one line midway between the antigen and serum basins The anti OM but not the anti pOM serum also showed a line close to the serum basin This pattern of precipitation lines was seen when OM was dissolved in the NaD buffer No line was observed when the preparations were suspended in PBS and tested

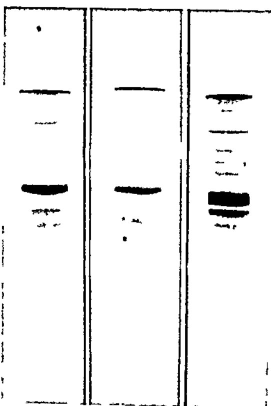


Fig 3 SDS polyacrylamide gel electrophoresis of the outer membrane from the *N gonorrhoeae* strains 8551 (left) V (middle) and VII (right)

Indirect Haemagglutination Testing

The anti OM sera contained antibodies both against LPS and the protein determinant *b* of the corresponding strains (Table 1) Antibody activity against determinant *b* was also shown by the anti pOM sera but they showed no antibody activity against LPS

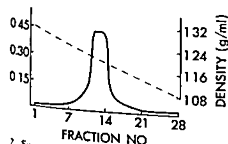


Fig 2 Sucrose gradient centrifugation of the outer membrane from the *N gonorrhoeae* strain VII Solid line indicates absorption at 280 nm and broken line density of sucrose



Fig 4 Double diffusion in agar gel with anti-OM 8551 (lower left) and anti pOM 8551 serum (lower right) against outer membrane from the *N gonorrheae* strain 8551 (upper well)

TABLE 1 *Titres of Anti OM 8551 OM V and OM VII Sera and the Corresponding Anti pOM Sera Against Erythrocytes Sensitized with LPS or Determinant* b *of the Homologous* N gonorrhoeae *Strains*

Antiserum	Antigen LPS	Det *b*
Anti OM 8551	256	512
Anti pOM 8551	<4	1024
Anti OM V	256	1024
Anti pOM V	<4	1024
Anti OM VII	256	2048
Anti pOM VII	<4	2048

LPS was oxidized with periodate and then used for the sensitization of erythrocytes Antibody agglutinating the erythrocytes was not present in any of the antisera examined

Antibody to the *b* determinant was removed by absorption of the antiserum with untreated or heated gonococci no matter whether homologous or heterologous bacteria were used Antibody activity to LPS in absorbed or cross absorbed antisera was not studied

Bacterial Agglutination with Anti pOM Serum

The antisera strongly agglutinated the homolo gous or heterologous heated gonococci in slide agglutination testing and in the plate agglutination test with the titres shown in Table 2 On absorption of any of the antisera with one or both of the heterologous strains only the homologous strain was agglutinated The agglutinating capacity of the antisera after cross absorption with both of the heterologous strains is shown in Table 3 This antibody activity could be eliminated by absorption with the homologous gonococci or OM preparation

When the antisera were tested against unheated gonococci only weak agglutination was observed and the formation of the agglutinates was delayed in comparison with that observed with the heat treated bacteria

TABLE 2 *Titre in Bacterial Agglutination with Anti pOM 8551 pOM V and pOM VII Sera*

Antiserum	Antigen 8551	V	VII
Anti pOM 8551	1024	512	512
Anti pOM V	128	256	256
Anti pOM VII	512	512	1024

TABLE 3 *Slide and Plate Agglutination with Cr Absorbed Anti pOM Sera Against the* N gonorrh *Strains 8551 V and VII*

Antiserum	Antigen 8551	V	VII
Anti pOM 8551	3 + (128)[a]	−(<4)	−(<4)
Anti pOM V	−(<4)	2 + (64)	−(<4)
Anti pOM VII	−(<4)	−(<4)	3 + (256

[a]) Denotes intensy of reaction in the slide agglutinat test with antiserum diluted 1 4 and in the paranthe titre in the plate agglutination test

The agglutination experiments were also perf med with gonococci grown without antibacter agents added to the culture medium Agglutinat similar to that described was obtained with th bacteria

Enzyme Digestion

Each of the OM preparations was digested w the various proteolytic enzymes The final lyoph zed preparation was then used for the absorption a portion of the corresponding anti pOM serum t had already been absorbed with the heterologo bacteria (see Table 3) Digestion with pronase bef the absorption destroyed the ability of all three O preparations to remove the antibodies agglutinati the corresponding gonococcal cells (Table 4) Peps digestion had a similar effect on the OM preparati from the strains 8551 and VII but not the strain preparation Papain or trypsin digestion did n destroy the antibody absorbing ability of the prep rations The results demonstrate susceptibility proteases of the OM antigen that functioned agglutinogen in tests with the cross absorbed ant pOM sera

Treatment of the OM preparations with perioda did not affect their antibody-combining ability

TABLE 4 *Slide Agglutination with Cross Absorbe Anti pOM Sera Against the Homologous Gonococcus aft Absorption of Antisera with Untreated or Enzym Digested Outer Membrane*

OM digested with	Anti pOM 8551	V	VII
Papain	−	−	−
Pepsin	2 +	−	2 +
Pronase	3 +	2 +	2 +
Trypsin	−	−	−
None	−	−	−

materials prepared from the *N gonorrhoeae* ıs with the EDTA buffer were greatly enriched embraneous structures that showed banding as gle peak at a density of from 1 18 to 1 23 g/ml crose in a gradient centrifuged at 105000 × g 8 h The

oresis dissociated into sub-units with a mole weight of around 12500 daltons (16) At least fferent proteins were present including a nating protein with sub-units about the same s that described by other investigators as the outer membrane protein of gonococci (7 8 These findings strongly indicate that the rations were greatly enriched in the outer brane of the bacteria However the possibility he OM preparations were contaminated with ns of a different origin such as cytoplasma brane proteins can not be excluded

ar gel precipitation showed that the anti-OM contained antibodies against at least three brane precipitinogens and the anti pOM sera t at least two of them Both categories of a contained antibodies to the protein determi that is probably produced by all gonococci y meningococci (13) Anti OM but not anti contained anti LPS antibodies This finding with earlier observations showing that ate treatment destroyed the antibody-combi tructures of gonococcal LPS (12) Nor was dy production against periodate-oxidized LPS strated in spite of the earlier observations e carbohydrate moiety of this component was mpletely disintegrated by the treatment (14) esults strongly support the notion that tion of antibodies to LPS can be avoided by on of the antigen with periodate prior to ization and that this treatment does not re with antibody production against perio-sistant antigens

anti pOM sera agglutinated the heat treated occi in accordance with earlier observations ng that surface proteins function as agglutino-n the gonococcus (1) The periodate resistant ns included agglutinogen(s) common to the 8551 V and VII and a seemingly strain c agglutinogen That this latter agglutinogen f protein nature was substantiated by its tibility to pronase and for the strains 8551 II to pepsin as well

dstrom & Danielsson (17) recently reported esults ... e study of gonococcal serotypes detected utilizing cross absorbed antisera in a coagglutination test They divided the gonococci into the antigen classes W J and M The heat resistant W class antigens were mostly of protein nature and were subdivided into the groups W/I W/II and W/III They showed that the strains VII and V used in this study belonged to the group W/I and W/II respectively and that strain 8551 reacted with the W/III as well as a W/II antibody reagent The strain specific antigens detected in the present study by direct agglutination of the bacteria may correspond to the group antigens of the W antigen class of gonococci Also the OM proteins characterizing the strains VII V and 8551 may correspond to the immunotypes A B and C detected by *Wang et al* (19) using mouse antisera to gonococci in a micro immunofluorescense test The present demonstration of serological diversity of proteins in gonococci is also consistent with results reported by other investigators (4 8 9) although it seems less clear how these results correspond to those of the present study

The experiments described were performed with gonococci grown in the presence of antibacterial agents to eliminate the growth of contaminating bacteria However when gonococci cultured wit hout antibiotics in the medium were used as antigen they showed the same results in the bacterial agglutination tests indicating that neither the common nor the strainspecific antigen was affected by the agents used

In studies not yet completed we have found that the majority of gonococcal isolates can be readily classified by slide agglutination using the cross absorbed anti pOM sera described in this study

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ANTIBODIES TO THE STRAIN-SPECIFIC AND CROSS-REACTIVE DETERMINANTS OF THE HAEMAGGLUTININ OF INFLUENZA H3N2 VIRUSES

2 *Antiviral Activities of the Antibodies in Biological Systems*

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Haaheim L. R & Schild G C Antibodies to the strain specific and cross reactive determinants of the haemagglutinin of influenza H3N2 viruses 2 Antiviral activities of the antibodies in biological systems Acta path microbiol scand Sect B *88* 335–340 1980

Antibodies to the strain specific (SS) and cross reactive (CR) antigenic determinants of the haemagglutinin (HA) of influenza H3N2 viruses were prepared and their antiviral properties in experimentally infected mice and in eggs were investigated The SS antibodies were considerably more efficient than the CR antibodies in direct virus neutralization tests The difference in the activities of the two types of antibody preparations was less pronounced in virus growth inhibition studies in the allantois-on shell system and in passive protection studies in mice given antibody before challenge with virus Nevertheless [illegible] ective than was CR [illegible] ntibody preparations [illegible] virus on day three post infection compared with the control animals The degree of reduction in titre exerted by the SS antibodies was greater than for the CR antibodies In contrast the rate of clearance of pulmonary virus 9–12 days after infection in the mice treated with SS antibody was slower than for the CR treated animals Also in SS antibody treated animals viruses isolated from mouse lungs showed evidence of minor antigenic variation away from the parent virus

Key words Influenza haemagglutinin strain specific antibodies cross reactive antibodies biological activity

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Antibody to the haemagglutinin (HA) antigen of influenza virus is considered to be an important factor in protection against infection (se reviews 8 3) The different antigenic determinants of the HA molecule of influenza A viruses can operationally be divided into the strain specific (SS) determinants which undergo frequent minor changes (antigenic drift), and the cross [illegible] ive (CR) determinants which are less [illegible] antigenically common for a range of viruses within a subtype (6 7 12)

We have previously shown that the serological potencies in single radial-diffusion tests and haemagglutination inhibition assays for antibodies to the SS and CR antigenic determinants differ markedly Antibodies to the SS determinants exhibited higher *in vitro* serological activity per µg IgG than did antibodies to the CR determinants a finding that could be due to the presence of more CR than SS determinants on the HA molecule (6)

The present report describes the anti viral activi ties of these antibodies in biological systems The activities of antibody preparations against the SS and CR determinants were investigated in virus neutralization tests in virus growth inhibition experiments in the allantois on shell (AOS) system and in challenge studies in mice passively given antibody before infection

MATERIALS AND METHODS

Virus Strains Immune Sera Strain specific and Cross reactive Antibody Preparations

All test strains sera and antibody preparations were as described previously by *Haaheim & Schild* (6) In brief immune goat serum to purified HA antigen from A/Hong Kong/1/68 (H3N2) virus was exhaustively absorbed with strains of the H3N2 subtype namely A/England/42/72 and A/Victoria/3/75 in order to obtain different preparations of strain specific anti HK/68 HA antibodies (SS and SS respectively) The cross reactive antibodies (CR and CR respectively) were isolated by eluting antibodies from the absorbing viruses A control goat serum free of anti H3 antibodies was prepared by the complete absorption of antiserum with HK/68 Eng/72 and Vic/75 viruses and subjected to DEAE cellulose chromatography in order to obtain a pure IgG preparation This material is referred to as Non HA IgG

HK/68 virus was adapted to become lethal to mice by five consecutive skim passages in mice according to *Fazekas de St Groth & Tees* (3)

Mice

Adult outbred female mice (NMRI/Bom) weighing between 20–22 g were used

Antibody Assays

Virus neutralization (VN) tests These were perform by mixing serial 10 fold dilutions of antibody prepa tions with 100 egg infective doses (50%) (EID_{50}) a incubating the mixtures for 30 min at room temperatu before inoculating allantoically into 6 eggs per antibo dilution The 50% virus neutralization end point w calculated (9)

Virus neutralization in mice was performed by mix equal volumes of serial 10 fold dilutions of antibody a 4 mouse lethal doses (50%) (MLD_{50}) of mouse adapt HK/68 virus The mixtures were incubated for 30 min room temperature and then kept at 0 °C while perfo ming intranasal inoculation into mice Five anaestheti animals were given 50 µl of each antibody virus mixtu Mortality was recorded and the experiments we terminated 10 days post infection

Growth inhibition studies Cultures of fragments chorio allantoic membranes attached to the egg sh (AOS cultures) were prepared as described by *Fazekas St Groth & White* (2) For growth inhibition tests ser 3 16 fold dilutions (½ $\log_{10}$ steps) of antibody we made and each AOS culture was challenged with 1 AOS_{50} doses of virus (5)

In the mouse system groups of 5 animals were give 0 2 ml volumes of each antibody dilution intraperit neally one day before challenge with 4 MLD_{50} of mous adapted HK/68 virus and mortality was recorded for 1 days post infection

In two independent studies groups of mice were give different goat IgG antibody preparations (2 µg IgG animal) or PBS one day before intranasal challeng with 0 1 MLD_{50} of virus Three to four animals we killed on days 3 6 9 and 12 post infection and th remaining animals were killed 20 days (Experiment I) o

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TABLE 1 *Direct Virus Neutralization Properties of SS and CR Antibody Preparations Obtained from Immune Goat Serum to Purified HA Antigen of A/Hong Kong/1/68 (H3N2) Virus*

Antibody preparation tested[a]	Indicator system for virus infectivity: Embryonated eggs		Mice	
	Log_{10} titre/ml	Spec act [b]	Log_{10} titre/ml	Spec act
SS	4 5	80	4 5	80
CR	2 9	4	3 4	12
SS	4 7	280	4 8	350
CR	4 2	30	4 4	45
Non HA IgG	<1 5	<1	ND[c]	

[a] SS (SS') represents residual (strain specific) antibodies after absorption with Vic/75 (Eng/72) virus whereas CR (CR) represents cross reactive antibodies eluted from the absorbing strain Non HA IgG is IgG fraction withou anti influenza HA activity

[b] Specific activity presented as ratio of geometric mean titre of duplicate tests to IgG concentration of antibody (µg/ml)

[c] Not done

TABLE 2 *Growth Inhibition Tests with SS and CR Antibody Preparations Obtained from Immune Goat Serum to Purified HA Antigen of A/Hong Kong/1/68 (H3N2) Virus*

AOS inhibition tests [a]

	SS	CR	SS"	CR"	Non HA IgG
Log_{10} titre (duplicate tests)	4!	3 4	3 8	4 0	<1 0
Specific activity (titre/µg IgG)	44	12	36	20	<1

Passive protection studies in mice (representative experiment)

SS µg IgG/animal	SS death rate	CR µg IgG/animal	CR death rate	SS µg IgG/animal	SS death rate	CR" µg IgG/animal	CR" death rate
39 8	0/5 [b]	21 3	0/5	18 3	0/5	16 9	0/5
12 6	1/5	6 7	1/5	5 8	0/5	5 4	0/5
4 0	0/5	2 1	3/5	1 8	1/5	1 7	1/5
1 3	1/5	0 7	5/5	0 6	2/5	0 5	2/5
0 4	5/5	0 2	5/5	0 2	5/5	0 2	4/5
IgG mouse protective-dose (50 %) MPD_{50} for duplicate tests							
0 9		4 0		1 0		1 0	

a) Allantois-on shell inhibition tests see Materials & Methods

b) Ratio of cumulative number of dead mice at day 10 post infection to number of test animals in the group given antibody intraperitoneally one day before challenge with 4 MLD_{50} of HK/68 virus

for haemagglutination inhibition (HI) antibodies against the strains HK/68 Eng/72 and Vic/75 by standard methods (16)

RESULTS

Direct Virus Neutralization Activity

Dilutions of antibody preparations were incubated with standard concentrations of virus (100 EID_{50} or 4 MLD_{50}) before inoculating into embryonated eggs or given intranasally to mice The results of these experiments are presented in Table 1 All antibody preparations showed evidence of neutralizing virus infectivity but there were marked differences in their specific activities (titre/µg IgG) The most active preparation was the narrowly reactive SS antibody (SS) whereas the most broadly reactive CR antibody (CR) showed the lowest specific activity The relative activities were similar to those found previously for *in vitro* assays (6)

Virus Growth Inhibition Activity

AOS inhibition tests These experiments were done in the AOS system where pieces of chorio allantoic membranes attached to egg shell were infected with 100 AOS_{50} doses of virus and maintained in medium containing dilutions of antibody The results of these assays are presented in Table 2 The difference between the specific activities of the various antibody preparations tested was smaller than in the neutralization experiments described above Even the most broadly reacting CR antibody (CR) performed well in this type of assay

Passive mouse protection tests Groups of mice [illegible]

conferred passive protection against fatal infection The experiments were performed in duplicate and the minimum protective dose of IgG necessary to inhibit death in 50 % of the animals was calculated As for the AOS inhibition tests the 50 % protective dose for these antibody preparations fell within a limited range the SS antibodies being about 4 fold more active per quantity of IgG than the CR antibodies

Effect of Passively Given Anti-HA Antibody on a Sublethal Murine Influenza Infection

Effect on pulmonary virus In order to investigate whether viral replication in mouse lungs was modified by the presence of non protective levels of passively administered anti HA antibody before infection animals were given 2 µg IgG of either SS CR or Non HA IgG others were given PBS only before challenge with 0 1 MLD_{50} of HK/68 virus (Experiment 1) In a duplicate test (Experiment 2) PBS was employed as a placebo The results of both experiments (Fig 1) were similar Firstly the greatest reduction of pulmonary virus titre on day 3 post infection was observed for mice treated with SS antibody Secondly both experiments showed that at day 9 post infection the

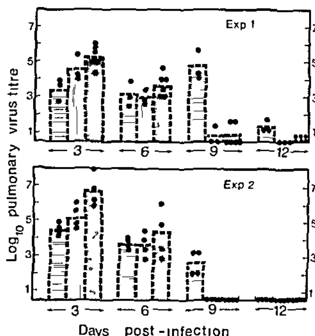


Fig 1 Geometric mean titres of pulmonary virus assayed in the AOS system in groups of mice given 2 µg IgG of SS antibody (horizontally shaded histograms) or CR antibody (vertically shaded histograms) intraperitoneally 1 day before challenge with 0 1 MLD_{50} of HK/68 virus The darker shaded histograms represent the placebo treated animals In experiment 1 (upper panel) the placebo group consists of animals given either PBS or 2 µg of Non HA IgG whereas in experiment 2 (lower panel) only PBS was employed as a control Individual titres are presented as black dots Titres <10 (log_{10} titre <1) located on or just above the abscissa are assigned the value of 5 when calculating mean titres

clearance of virus from the lungs were delayed in animals treated with SS antibody

Viruses isolated on day 9 post infection we assayed in HI tests with the original goat serum purified HK/68 HA antigen and the two anti subfractions SS and CR (Table 3) Viruses isolate *from the SS treated animals* showed evidence minor antigenic drift away from the parent HK/6 virus when tested with SS antibody (>four fo reduction in HI titres compared with the pare virus) and from the viruses isolated from the CR treated and placebo treated animals HI tests w anti HK/68 HA serum or with CR antibody d not reveal evidence of antigenic differences the strains

Effect on post infection HI antibodies It has bee observed in man (10 14) that inactivated influenz vaccines administered to primed individuals posse sing prevaccination CR antibody to H3N2 viru rarely evoke SS antibodies to the vaccine virus possible explanation for these observations is th the CR antibody may have a blocking effect on th expression of *SS* determinants It was therefore c interest to see whether passively administered CI or SS antibody interfered with the anti HA antibod response in experimentally infected mice Ou experiments described above revealed no effect c this kind Sera collected from the various treatmen groups of mice on day 20 or 28 post infection al had comparable levels of post infection *HI* antibo dies to both the homologous HK/68 virus and th heterologous H3N2 variants Eng/72 and Vic/7 (data not shown) Control mice given antibody bu not infected had low or undetectable levels of serum HI activity throughout the experiment Thus i appears that the amount of passively administere

TABLE 3 *HI Tests with Pulmonary Virus Isolated on Day 9 Post infection with A/Hong Kong/1/68 (H3N2) Virus and Goat Antiserum to Purified HA Antigen of HK/68 Virus and Two Antibody Subfractions CR and SS*

Mice treated with	Virus isolates tested	Antibody preparations		
		HK/68 goat serum	CR	SS
SS antibody	511	12 0[a]	7 1	6 3
	512	11 3	7 3	6 1
CR antibody	530	12 8	8 1	7 6
	531	13 3	7 6	8 6
	532	13 0	7 4	8 3
PBS (control)	552	13 1	7 6	7 9
	553	12 8	7 4	8 3
	554	13 3	7 9	7 8
	574	13 6	8 4	8 1
	A/HK/1/68	13 1	7 9	8 6

a) Log_2 HI titre of geometric mean of duplicate tests

CR antibody given to mice in our experiments
measureable effect on the expression of the
s antigenic determinants on the HA molecule
infecting virus

DISCUSSION

ave previously found that the *in vitro*
gical properties of SS and CR anti HK/68
ntibodies obtained from immune goat serum
d markedly The most narrowly reacting SS
dies (SS") exhibited higher specific activity per
ity of IgG than did the most broadly reacting
ntibody (CR) (6) Our observations have now
extended to include their antiviral properties
ling d rect virus neutralization passive protec
of mice against lethal challenge inhibition of
growth in AOS cultures and effect on
nary virus growth in mice after a sublethal
challenge

the present studies using direct VN tests *i e*
d exposure of virus to antibody *in vitro*
oying embryonated eggs or susceptible mice as
ator systems for virus infectivity considerable
ences were detected in their specific activities
SS" antibodies being about 30–70 fold more
ive than the CR antibodies This difference in
fic activity was greater than that detected
een these preparations in HI tests (6) However
lifference in specific activities of the antibody
less pronounced in virus growth inhibition tests
e AOS system or in passive protection of mice
st lethal challenge Nevertheless even in these
riments the SS antibodies were somewhat
e effective (3–4 fold) than the CR antibodies
results of our mouse protection studies using
ively administered antibodies were in accor
e with those of *Virelizier* (11) who used
enza A viruses of the H0N1 H1N1 subtype
observed better protection by strain specific
HA antibod es than by the cross reactive
bodies

N assays and passive mouse protection assays
an obvious relevance to our understanding of
logical parameters of immunity to influenza In
ion AOS inhibition tests and titration of
nonary virus in infected mice are biologically
vant in that they provide information on the
ty of antibody to modify viral replication once
ction is established When mice were passively
h small quantities of narrowly reacting strain
cific (SS") anti HA antibody before challenge
h a sublethal dose of HK/68 virus pulmonary
s titres were reduced on day 3 post infection
pared with the control mice A lower degree of
uction was de ... th CR antibody Howe

ver while control animals showed a normal clearance of pulmonary virus 9–12 days after infection (1 17) an unexpected finding was that the SS" antibody treated animals showed a reduced clearance rate with virus being detectable on post infection day 12 In addition virus showing evidence of minor antigenic variation could be isolated from these animals In another report (4) we present evidence that both SS and CR antibody preparations will select antigenic variants in the murine system but that the SS" antibodies do so more efficiently than CR antibodies

Little is known about the characteristics of serum anti HA antibodies which are most effective in conferring protection against influenza in man but our present results may have a bearing on the human situation Our findings in mice imply that solid resistance to infection might best be provided by antibodies to the SS antigenic determinants of the HA molecule of the infecting virus However this type of antibody might be of more limited value in the natural protection against influenza in man because of the frequent antigenic drift in the HA antigens of the prevalent viruses High levels of CR antibody might also confer some protection and against a wider range of antigenic variants Once infection is established the SS and CR antibodies appear to interfere with viral replication to very similar degrees CR antibodies may be more effective in this situation because of their broad reactivity with variant influenza viruses within a subtype and because they appear to be less effective in selecting new antigenic variants

It has been shown (10 14) that inactivated influenza A (H3N2) vaccines used in antigenically primed human adults in the interpandemic period may stimulate mainly CR antibodies and SS antibodies directed against the first member of the H3N2 subtype (A/HK/68 virus) rather than against the vaccine virus In contrast in previously unprimed children who received similar vaccine SS *antibody against the vaccine* virus was frequently induced (*Oxford et al* in preparation) Taken together with information on the differences in the biological activities of the CR and SS antibodies presented here these findings may be relevant to the understanding of the often incomplete and variable degrees of protection associated with the use of inactivated influenza vaccines in man

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haemagglutinin (HA) antigen from HK/68 virus and various subfractions of anti-HA populations of antibody were prepared as described previously (5) In brief, the goat serum was exhaustively absorbed with concentrates of H3N2 variants A/England/42/72 and A/Victoria/3/75 in order to obtain different preparations of strain specific anti-HK/68 antibodies (SS'' and SS', respectively) and cross-reactive antibody preparations (CR'' and CR', respectively) Post-infection ferret sera were prepared as described earlier (4)

Mice

Adult outbred female mice (NMRI/Bom), weighing between 20–22 g, were used

Isolation of Variants

In allantois-on-shell The methods described previ ously were followed (4), except that antibody was added to the growth medium in a concentration 3–5 times as high as that required to inhibit growth of 500–1000 AOS infective doses of virus in 50% of the cultures

In mice The IgG-dose of each antibody preparation required to inhibit death in 50% of mice challenged with four mouse lethal-doses (50%) of virus, 4 MLD_{50}, was determined as described before (6) In experiments where we looked for antigenic variants of HK/68 virus in mouse lungs, animals were given five times this IgG amount intraperitoneally and challenged intranasally with 0 1 MLD_{50} of virus on the following day For each [illegible]

obtained at the dilution end-point was subjected to another egg passage at limiting dilution before being tested serologically Also strains isolated in the AOS system were cloned twice in eggs before use

Serological Tests

Haemagglutinations-inhibition (HI) tests and single radial haemolysis (SRH) absorption tests were done as described in (13) and (8) respectively Briefly, the SR absorption assays were done as follows SRH plat containing HK/68 virus or antigenic variants, we prepared Three 20 µl aliquots of post infection fer serum were mixed with equal volumes of eith *homologous, heterologous or control virus concentrate* the control virus being A/chicken/Germany/'N/ (Hav2Neq1), and left at room temperature for 1 followed by 1 h at 4 °C, before testing for resid antibody in both homologous and heterologous SR plates Lytic zones were measured by use of a calibrate viewer (Diffusion Zone Reader, Dynatech, Switzerland

RESULTS

Apparent Frequency of Isolation of Antigenic Vari ants

In the course of this study it became evident th the various anti-HK/68 HA antibody preparation differed in their ability to select new antigeni variants This is illustrated in Table 1 Both in th AOS system and in mice the most narrowl reacting SS antibody preparation (SS'') exhibited th highest apparent ability to select new strain showing evidence of 'antigenic drift' in the HA antigen However, the criterion for being a antigenic variant was that the isolated strains shoul *react ≧2-fold lower than the HK/68* virus in H tests with the parent post-infection ferret serum The occurrence of so-called 'non-avid' strains giving low HI titres in general (1), will obviousl also score as an 'antigenic variant' in Table 1 These data should therefore be considered as describing the relative frequencies of appearance of new antigenic variants For the *in vitro* and *in vivo* experiments taken together, the SS'' antibodies seemed to provide the most successful growth advantage for antigenic

TABLE 1 *Frequency of Isolation of HK/68 Variants giving ≧ 2 fold Reduction of HI Titres in Tests with Post-infection Ferret Serum to HK/68 Virus in Parallel Assays with Parent Virus*

Selecting antibody[a]	Frequency of variants		
	In vitro (AOS)	*In vivo* (mice)	Total
SS'	14/40[b]	2/25[c]	16/65 (25%)
CR'	5/33	3/24	8/57 (14%)
SS	11/34	14/25	25/59 (42%)
CR''	14/49	9/25	23/74 (31%)

a) SS' (SS) represents residual (strain specific) antibodies after absorption with the heterologous H3N2 strains A/Victoria/3/75 (A/England/42/72), whereas CR (CR) represents cross reactive antibodies eluted from the absorbing strains

b) Ratio of number of strains being possible antigenic mutants to number of virus-positive AOS cultures

c) Ratio of number of strains being possible antigenic mutants to number of virus positive mouse lungs (pairs) investigated

TABLE 2 *Antigenic Relationships between A/Hong Kong/1/68 (H3N2) and Variants Obtained* in Vitro *and* in Vivo *in HI Tests Performed with Post infection Ferret Sera*

In vivo variants							*In vitro* variants					
Virus strains	Antiserum to						Virus strains	Antiserum to				
	HK/68	SS 4B	CR 4E	SS 3E	CR 3D			HK/68	SS 4	CR 1	SS 22	CR 11
HK/68	*10 32*[a]	9 61	9 32	8 82	9 32		HK/68	*10 32*	9 61	9 32	9 61	8 32
SS 4B	9 32	*10 11*	9 61	8 82	8 91		SS 4	8 82	*9 61*	8 82	9 11	7 32
CR 4E	8 82	9 32	*10 32*	9 82	7 61		CR 1	8 32	8 32	*9 32*	8 61	9 11
SS 3E	9 11	7 82	8 82	*9 61*	9 32		SS 22	8 61	8 61	8 82	*9 82*	8 91
CR 3D	8 32	6 82	6 82	8 82	*9 11*		CR 11	9 32	8 11	8 11	8 32	*10 11*

[a] Log_2 geometric mean HI titre of duplicate tests

mutants the frequency being about 3 fold higher for the SS than for the CR antibodies The corresponding frequencies for the two other anti body preparations the more broadly reacting SS and the less cross reactive CR antibodies were of intermediate magnitude

For the experiments in mice we also found that out of a total of 28 variants showing reduced HI titres with post infection ferret serum to HK/68 virus, 20 were isolated on days 3 and 4 post infection Only SS treated animals gave influenza isolates being possible antigenic mutants on day 5 post infection

Antigenic Relationship between Variants and Prototype Virus

HI tests In Table 2 are presented HI data for one set of representative antigenic mutants obtained for each antibody preparation in tests with post infection ferret sera For sera against mutants obtained both *in vitro* and *in vivo* there was a general tendency to give higher HI titres with the homologous than with the heterologous strains Furthermore by comparing the HI data for HK/68 virus and each mutant in tests with their respective sera the HK/68 serum usually distinguished more clearly between parent and mutant strain than did each mutant serum This is known as an asymmetrical reaction pattern (1) None of the mutant sera exhibited reaction patterns resembling that of the HK/68 serum thus indicating that these isolates were in fact antigenic variants of HK/68 virus and not merely non avid strains Although all HI titrations were done in parallel and in duplicate with all dilutions of sera made up in bulk before transfer to titration plates and with the use of the same suspension of erythrocytes the HI method is a rather inaccurate method of measuring antigenic similarities between related strains

SRH absorption tests These assays were done in

TABLE 3 *Antigenic Relationship between A/Hong Kong/1/68 (H3N2) and Variants Obtained* in Vitro *and* in Vivo *in SRH Cross absorption Tests with Post infection Ferret Sera*

In vivo variants				*In vitro* variants		
Variants tested	HK/68 serum absorbed with variant tested in HK/68 plate	variant serum absorbed with HK/68 tested in variant plate		Variants tested	HK/68 serum absorbed with variant, tested in HK/68 plate	variant serum absorbed with HK/68 tested in variant plate
SS 4B	19[a]	10		SS 4	7	26
CR 4E	29	11		CR 1	5	25
SS 3E	35	26		SS 22	9	16
CR 3D	21	3		CR 11	2	31

[a] Per cent of ... g in comparison with zone area after absorption with control virus A/chicken/

order to provide a more precise picture of the antigenic relatedness between mutants and parent virus Post-infection ferret sera were exhaustively absorbed with concentrates of either homologous, heterologous or control ('N') viruses before testing for residual antibodies in homologous and heterologous SRH plates As all homologous absorptions were complete, *i e* no lytic zones could be detected, only the results of the heterologous absorptions are shown in Table 3 and presented as per cent of lytic zone area remaining in comparison with area of control zone Only infrequently were anti-neuraminidase antibody zones observed around the control wells, but their larger and weaker appearance made them easily distinguishable from the clearer and sharper anti HA zones For the *in vivo* mutants it was demonstrated that a significant proportion of antibodies to the HA antigen could not be absorbed by heterologous virus There was also a tendency for the mutants to absorb the anti-HK/68 antibodies less completely than in the *vice versa* type of experiments Similar patterns of cross reactivity were seen for the *in vitro* mutants, although for these strains the anti-HK/68 antibodies were almost completely absorbed by the mutant strains

DISCUSSION

We have described which specificity of antibody to the HA antigen of A/Hong Kong/1/68 (H3N2) virus, obtained from immune goat serum to purified HA antigen, seemed to have the property of selecting antigenic mutants by allowing the parent virus to multiply in the presence of either SS of CR antibody preparations Our results clearly indicate that all antibody preparations tested have this property both *in vitro* (AOS) and *in vivo* (mice) The most narrowly reacting SS antibody preparation (SS) showed the highest capacity to select variants showing antigenic drift in the HA antigen whereas the most broadly reacting type of antibody (CR) provided less favourable conditions in this respect However, the true capacity to select new antigenic variants cannot be resolved without preparing antisera to all possible antigenic variants Assuming, however, that the occurrence of non avid strains was equally frequent for all antibody preparations tested, the overall data presented in Table 1 indicate that the SS antibodies are the most effective for selective growth of new antigenic variants

On the basis of our previous data (6), suggesting that the SS antibodies exhibit a higher neutralization capacity per molecule of IgG, both in the AOS system and in experimentally infected mice, it was not unexpected that the specific antibodies seemed [to] provide the most successful selective growth con[ditions] for new antigenic variants This has also be[en] suggested by *Virelizier et al* (10)

However, all antibody preparations tested had t[he] ability to provide selective growth advantages f[or] new antigenic variants with changes in the H[A] antigen The data presented in Table 2 showed th[at] the various strains tested were different from t[he] parent HK/68 virus based on the finding th[at] antiserum to the parent virus reacted different[ly] with the mutants than did the various mutant ser[a] This difference in antigenic profile between pare[nt] and each mutant may seem trivial, but this is to b[e] expected when single-cycle mutants are teste[d] (*Haaheim & Schild* (4)) Furthermore, the cros[s] absorptions experiments in SRH plates also showe[d] that the reciprocal ability to absorb heterologo[us] antibodies was not complete, confirming that a[n] antigenic difference existed between the parent an[d] each mutant (Table 3) Both for the *in vitro* and *i[n] vivo* variants there was an asymmetrical pattern o[f] absorption of heterologous antibodies, although th[e] two groups of antigenic variants differed in th[is] respect However, the two methods of selectin[g] antigenic mutants varied considerably in experimen[tal] design so that a straightforward compariso[n] between observations may not be justified Even so it is difficult to provide a reasonable explanation fo[r] this discrepancy

The apparent frequency of isolation of variants seemed high for all antibody preparations tested In view of recent works by *Yewdell et al* (14) and *Lubeck et al* (7) suggesting that the frequency of isolation of antigenic variants employing mouse monoclonal antibodies to non overlapping antigenic sites on the HA antigen is in the order of 10^{-5} our data with polyclonal antibody preparations seem to be in conflict with theirs Irrespective of how well defined the specificity of our goat antibody preparations may appear they probably comprise a range of antibody clones with varying specificities and affinities It has been estimated that the probability of obtaining a double mutant, *i e* a variant with changes at two different antigenic loci by the use of two different monoclonal antibody populations in one single experiment should be in the order of about 10^{-10} per infectious unit of virus (12 14) Not surprisingly, such single step double mutants have not been isolated experimentally using high concentrations of monoclonal antibodies in the AOS system (14) However in our study we have used relatively dilute and carefully balanced concentrations of antibody Consequently, only the most abundant clones or clone, of antibody would be expected to contribute to the selection process Also

me of these antibodies were directed against lapping antigenic sites the frequency of isolation itigenic variants will not be the product of the abilities of mutations at each of the sites gnised by these antibodies

e have in another study *Oxford et al* (in prep ion) found that children have more SS antibo to current influenza strains than adults who he other hand have more CR antibodies as well epidemiologically irrelevant SS antibodies to ious variants within the HA antigenic subtype SS antibodies of young children may therefore sibly provide the best selective growth conditions new antigenic variants Furthermore we have ntly tested some post-epidemic human sera inst an antigenic mutant of the latest epidemic N2 variant A/Texas/1/77 obtained by passage virus in the AOS system in the presence of a use monoclonal anti HA (Texas) antibody prepa on (3) Not unexpectedly we found that sera n young children reacted to lower HI titres with mutant virus than did sera from adults These ervations for human sera supplement the fin gs of the present report Our main conclusion refore is that the SS antibodies to the HA igen will provide the best selective conditions for wth of antigenic variants

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BRIEF REPORT

DEOXYRIBONUCLEIC ACID IS A SIGNIFICANT COMPONENT OF THE SMALL INTESTINAL MUCUS

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rencz A Orskov I Orskov F & Klemm P Deoxyribonucleic acid is a significant component of e small intestinal mucus Acta path microbiol scand Sect B 88 347–348 1980

uring investigations into the role of intestinal mucus for the attachment of *Enterobacteriaceae* to the testinal surface it was surprisingly found that DNA was a significant component of the mucus from e rabbit small intestine

ey words Small intestinal mucus DNA

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: attachment of bacteria to cells and mucous nces of human and animal origin has been studied r laboratory (9 10) As part of this series of iments we initiated investigations of attachment of ia to mucus from the small intestine of rabbits hough many investigators stress the importance of ole of the small intestinal mucus for intestinal uzation (5 6 11) its chemical composition has not well examined In the following we describe highly us DNA as a significant part of this material

hite rabbits weighing 3 5–4 0 kg were sacrificed he small intestines were taken out and placed on ice were cut into pieces of 15 cm and the mucus was ly pressed out gathered in a beaker on ice and kept jejunal and ileal mucus No obvious differences een these preparations could be found The mucus a strong tendency to adhere to glass and polyethy

he mucus was diluted into 12 volumes of ice-cold sphate buffered saline (PBS) conta n ng 100 . .

hours After centrifugation of the suspended mucus (830 g for 10 min) a very compact pellet was formed which had approximately the same volume as the initial undiluted mucus A thin layer of cells and bacteria was removed from the surface of the pellet by washing The main part of the pellet consisting of a network of fibres could be stained with toluidine blue at pH 7 4 which is known to stain not only glycoproteins but also DNA The lowest part of the pellet contained food particles and other materials such as sand and straw These particles were attached firmly to the network of fibres in the pellet Like the undiluted mucus the pellet was difficult to handle because of its extreme viscosity and centrifuga tion of the homogenate at higher g values only resulted in a compression of the pellet, making it even more difficult to handle

The pellet was washed 5–10 times with ice-cold PBS and dissolved by addition of 4–6 volumes of 0 33 M NH_4OH This procedure resulted in an extremely viscous solution from which the food remnants and other particles could not be removed

Precipitation of the dissolved material could be done in two ways by addition of PCA to a final concentration of 0 3 M or by addition of 3 volumes of 96 % ethanol The latter treatment resulted in the rapid appearance of long fibres which immediately aggregated to form a

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TABLE 1 *DNA RNA and Hexosamine Content (µg/mg Protein)ᶠ of the Small Intestinal Mucosa and Mucus in Rabbits*

	Small intestinal mucosa[a]	Small intestinal mucus[b]	
		Supernatant	Pellet
DNA[c]	8 0	24 0	519 4
RNA[d]	54 8	40 0	24 0
Hexosamines[e]	3 8	1 9	4 3

[a]Mucosa from the wall of the small intestine was obtained by the method of *Forstner* (4) The mucosal suspension was precipitated by 0 3 M PCA and washed twice with 0 3 M PCA extracted twice with 96% ethanol once with ethanol ether 1 1 and twice with ether The extracted sediment was dried at 37 °C and dissolved in 0 5 M NaOH for further processing

[b]To 3 ml of intestinal mucus 36 ml of PBS containing 100 µg/ml TI was added Homogenization was done by repeated pipetting with a wide tipped pipette The suspension was centrifuged at 830 *g* for 10 min at 0 °C The supernatant was collected and PCA was added in aliquots to a final concentration of 0 3 M Further processing was as described above for small intestinal mucosa The pellet was washed 8 times with PBS and dissolved in 8 ml 0 33 M NH_4OH and 3 volumes of 96% ethanol were added The resulting fibres were extracted with ethanol ethanol ether and ether as above The extracted fibres are designated › pellet ‹ 1 mg of the dried material was dissolved in 1 ml of 0 5 M NaOH for further processing

[c]DNA content was estimated by the diphenylamine reaction (2) and a spectrophotometric method (12) These two methods gave identical results within the limits of the experimental error

[d]RNA content was estimated according to *Fleck & Begg* (3)

[e]Hexosamine content of the hydrolysed samples was determined with a Durrum D 500 amino acid analyser as described earlier (8) Quantification was made relative to glucosamine

[f]The protein content was estimated by the method of *Itzhaki & Gill* (7)

Bovine serum albumin was used as a standard and the results were corrected for TI content in the small intestinal mucus supernatant

All operations were carried out at 0 °C Standard deviation of the results did not exceed ± 2%

clump that entrapped the above mentioned particles The DNA RNA protein and hexosamine content of these fibres and of the supernatant collected after the first centrifugation of the mucus was examined and compared with the content found in the mucosa of the small intestinal wall (Table 1) While the values in the three columns do not differ much from each other as regards RNA and hexosamine the high amount of DN in the pellet was unexpected and remarkable Th nature of the ethanol precipitated material was t various methods Resistance to alkaline hyc sensitivity to hydrolysis with 0 5 M PCA at 70 °(min as measured by increase in absorption at 2 and a positive diphenylamine reaction of tl hydrolysate all indicated the presence of DNA F more the substance was sensitive to crys pancreatic DNase I in the presence of Mg^{2+} ions

In repeated experiments 56–88% of the DN found in the pellet after the first centrifugation ; was difficult to make bacterial adhesion exper with this highly insoluble DNA material it has been possible to draw any conclusion about the ac of fimbriated or non fimbriated *Escherichia coli* DNA rich mucus

We do not know the origin of DNA in the intestinal mucus An alimentary origin of such amount of DNA is unlikely as is the bacterial because of the very few bacteria in the mucus tempt ng explanation is that the DNA originates fr intestinal mucosal cells which are disrupted after release from the villi and then forms a ne supporting the other components of the small int mucus Careful morphological and autoradiogra studies by *Abrams et al* (1) of the small int mucosal cell DNA labelled with tritiated thymidin shown that the turnover of the mucosal cells in the is a rapid process and that the movement of the generated cells progresses rapidly from the crypts luminal ends of the villi However the authors g data on the fate of the labelled DNA after release cells from the villi

We believe that the demonstration of a consid amount of DNA as a part of the small intestinal mu important for our understanding of the role played l small intestinal mucus in health and disease

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BRIEF REPORT

COMPARISON OF THE SENSITIVITY OF ELISA AND THE HAEMAGGLUTINATION INHIBITION TEST FOR ROUTINE DIAGNOSIS OF RUBELLA

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Vejtorp M & Leerhoy J Comparison of the sensitivity of ELISA and the haemagglutination inhibition test for routine diagnosis of rubella Acta path microbiol scand Sect B 88 349–350 1980

A total of 500 paired sera were tested for rubella antibodies by the rubella IgG ELISA and the haemagglutination inhibition (HI) test Significant differences between the antibody level in the first and second samples of 417 serum pairs were not detected by either of the tests whereas significant increases in 71 pairs were detected by both methods A significant increase of the level in 12 serum pairs was demonstrated by the ELISA but not by the HI test The rubella IgG ELISA is more sensitive than the HI test for diagnosis of recent rubella

Key words Rubella routine diagnosis ELISA HI

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…major problem in the serodiagnosis of recent …la by the haemagglutination inhibition (HI) test is …p d increase of specific antibody titers following the …tion Thus the detection of a diagnostic increase of HI titer may be missed if the first sample is collected …a few days after the rash In a previous study of sera …a small number of patients a slower increase was …ved when the rubella specific IgG antibodies were …mined by the enzyme linked immuno-sorbent assay …ISA) It was therefore presumed that the diagnostic …tivity i e the reliability of a negative test result (7) …ht be increased if the HI test was replaced by this …y (6) A comparison of the two methods applied for …ine diagnosis of rubella is reported in the present …mmunication

…erials and Meth…ds

A total of 500 paired sera submitted for routine …gnosis from patients with rashes exposed to or …cinated against rubella were tested The sera were …eived during a period of 3 months from general …itioners and hospital departments who also provi… …f the clinical information The sera were tested by the … test and the rubella IgG ELISA as previously …scribed (2 3 4) …hat a control antigen was not used in the ELISA and the results were read on a Titertek Multiskan® photometer The reported E values are the means of double determinations of single serum dilutions The test was repeated if the difference between the two determinations of one serum exceeded 15% An E value of 0 20 was used as the lower limit for the presence of antibodies

Differences between the antibody levels in the paired sera were considered significant if the HI titer increased four fold or more or if the E values increased 20% or more provided the difference between the two values was more than 0 05 and antibodies were detected in the second sam… … only … rubell… described (6)

Results

Significant differences between the levels of rubella specific antibodies in the first and second of 417 of the 500 pairs of sera were not detected either by the HI test or the IgG ELISA In 324 of these pairs rubella antibodies were detected by both methods whereas 84 did not contain specific antibodies Rubella IgG was demonstrated in 7 paired sera by ELISA but not by the HI test the E values being 0 20–0 35 (median 0 31) The

HI titer of one pair of sera containing rubella IgG could not be determined owing to natural agglutinins still present after the pre treatment The E values of one pair of sera with an HI titer of 10 were 0 17 and 0 18 respectively

The coefficient of variation (standard deviation/mean) calculated from the difference between the first and second of the 417 samples was 17% of E values less than 0 20 7% of E values between 0 21 and 0 50 and 4 5% of E values higher than 0 50

Significant increases of the rubella antibody levels between the first and second of 71 paired sera were detected by ELISA and the HI test The rise of the rubella IgG E values were 0 17–0 59 (median 0 35) corresponding to increases of 103–1680% (median 540) of the first value The increases of the HI titers were 2–8 (median 5) of two fold titration steps In each of 56 paired sera the first sample was collected 2 days after a rach or earlier whereas the first sample of 2 3 and 2 paired sera were collected on days 3 4 and 5 respectively One pair was received from a patient exposed to rubella but without a rash and one pair was collected before and after vaccination against rubella

A significant increase of the rubella antibody concentration from the first to the second of 12 paired sera was demonstrated exclusively by the ELISA Of these 11 pairs were collected from patients with rashes (Fig 1)

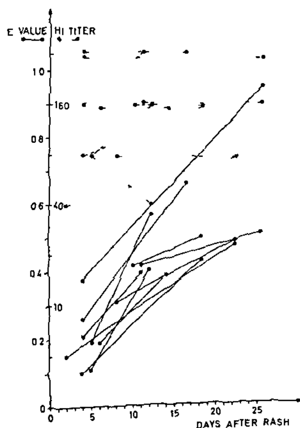


Fig 1 Results of testing 11 paired sera in which a significant increase in concentration of specific antibodies was detected by rubella IgG ELISA but not by the HI test

The increases of the E values were 21–333% (m 156) All samples contained rubella specific antibodies detected by ELISA except one obtain days after a rash Two sera which originated fr patient before and after rubella vaccination cont rubella IgG in both samples (E values 0 25 and but not rubella IgM antibodies

Discussion

The discrepancy between the HI test and the ru IgG ELISA was most marked in tests of sera of w the first had been obtained more than a few days af rash Thus 10 of 11 paired sera showing signif increases of the rubella IgG E values but not of th titers were collected 4 days after a rash or later (Fi as opposed to only 5 of the 63 sera in which signif increases were detected by both techniques The cause of the different results of the two method therefore probably the rapid development of the antibody titers compared to the rubella IgG E va after rubella infections This early increase in the titers is probably due to the contribution of specific antibodies which are not detected by the rubella ELISA The previously demonstrated higher precisio the ELISA technique (4) may further contribute to difference of the results

An alternative method for serodiagnosis of re rubella is the detection of specific IgM antibodies ELISA The assay may be applied routinely but is r complex than the IgG assay owing to the interferenc IgM rheumatoid factor which necessitates determin of the concentration or removal of this anti IgG e g absorption to aggregated IgG (5) A new EL technique which uses anti human IgM attached to a s phase may be less affected by the presence of rheumatoid factor (1)

In a previous comparison of the ELISA and the test for detection of rubella antibodies the hi sensitivity the ease of performance and possibilities automation of ELISA were emphasized (4) The pre study also showed a higher diagnostic sensitivity of rubella IgG ELISA

This study was supported by a grant from *Foundation of the Danish Health Insurance Societ* excellent technical assistance of Miss *Karin Hanse* gratefully acknowledged

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BRIEF REPORT

AEMAGGLUTINATION INHIBITION ANTIBODIES IN HUMAN SERA TO AN ANTIGENIC MUTANT OF INFLUENZA A/TEXAS/1/77 (H3N2) VIRUS OBTAINED *IN VITRO*

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aaheim L. R Haemagglutination inhibition antibodies in human sera to an antigenic mutant of ifluenza A/Texas/1/77 (H3N2) virus obtained *in vitro* Acta path microbiol scand Sect B 88 51–353 1980

in antigenic mutant of the wild influenza strain A/Texas/1/77 (H3N2) was obtained *in vitro* by rowing virus in the allantois-on shell system in the presence of a mouse monoclonal antibody reparation to the Texas haemagglutinin (HA) antigen A total of 204 human sera from a population of randomly collected sera during the fall of 1979 were examined by the haemagglutination inhibition HI) tests against the Texas wild strain and the antigenic mutant It was shown that sera from young children (0–5 years) reacted poorly or not at all with this mutant strain whereas sera from adults (15 years+) reacted equally well with both viruses It can be assumed that the antigenic site recognised by his particular monoclonal antibody preparation (185/1) may be important for the human immune response to the HA antigen of A/Texas/1/77 virus

Key words Influenza antigenic mutant, monoclonal antibody human sera

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onoclonal antibodies against the haemagglutinin antigen of various influenza viruses produced by atic hybrids between mouse myeloma cells and ed spleen cell (7) are now available (6 13 15) The of the present study was to determine whether an sera would inhibit viral haemagglutination in with the wild influenza strian A/Texas/1/77 N2) and a antigenic mutant obtained *in vitro* by vating A/Texas virus in the allantois-on shell em in the presence of monoclonal antibody against HA antigen In particular we wanted to see whether from various age groups differed in their reaction erns with these two virus strains

erials and Methods

1 *Virus strains* The preparation and antigenic racterization of antigenic mutants of A/Texas/1/77 N2) virus by the use of mouse monoclonal antibody he HA antigen of A/Texas virus will be described in a l elsewhere (*Haaheim et al* unpiblished) In brief us was cultivated in the allantois-on shell (AOS)

(Mo12) obtained through AOS passage of A/Texas virus with monoclone 185/1 Post infection ferret serum to A/Texas virus gave 8 fold lower haemagglutination inhibition titre with the mutant strain than with the homologous variant whereas antiserum to Mo12 reacted to similar titres with both strains In contrast the monoclonal antibody preparation used in our selection experiments gave a haemagglutination inhibition titre <50 with the mutant virus while having a titre of about

[illegible]

nated in embryonated hens eggs (8) so that the N2 neuraminidase antigens were replaced by the Neql antigenic subtype The recombinants will be called Tex and R12 respectively

2 *Human sera and serological tests* Sera were obtained from the [illegible]

[illegible] procedures (14) except that the assays were performed in microplates employing 50 µl volumes of each reactant All sera in this study were among those having HI titres ≧10 against the A/Texas prototype virus

TABLE 1 *Haemagglutination inhibition (HI) Reactions for Human Sera in Tests with A/Texas/1/77 (H3N2) and Antigenic Mutant Selected in Vitro*

Age groups (years)	No of sera tested	Virus strains	No of sera with stated HI titres			GMT[a]	Ratio GMT Tex/R12	No of sera (%) with ≧4 fold lower HI titre with R12 than with Tex
			<10	10–20	≧40			
0–5	30	Tex[b]	0	6	24	62 1	3 3	20 (67)
		R12[c]	8	12	10	19 1		
6–14	49	Tex	0	22	27	29 3	2 1	14 (29)
		R12	15	22	12	14 1		
15–24	39	Tex	0	9	30	46 1	1 4	1 (3)
		R12	0	16	23	33 5		
25–59	46	Tex	0	16	30	34 9	1 2	1 (2)
		R12	1	22	23	29 2		
60 +	40	Tex	0	13	27	38 0	1 1	5 (13)
		R12	5	16	19	33 2		

[a] Geometric mean HI titre Titres <10 were assigned the value of 5 when calculating GMT
[b] A/Texas/1/77 recombinant with Neql neuraminidase
[c] Mutant Mo12 recombinant with Neql neuraminidase

Results and Discussion

In all 204 post epidemic human sera from all age groups were assayed in HI tests with Tex and R12 viruses Table 1 summarizes the main results of our study For sera obtained from children aged 0–5 years the geometric mean HI titres (GMT) in tests with the two strains differed markedly (3 3 fold lower for R12) Also sera from older children (6–14 years) showed a clear fall in GMT (2 1 fold) whereas sera from adults (15 years +) reacted almost equally well with both strains Twenty out of 30 sera (67 %) from the youngest age group had HI titres ≧4 fold lower with R12 than with Tex virus Of the sera from adults only 7 out of 125 had this reaction pattern Again sera from older children showed an intermediate pattern of reactions with 14 out of 49 sera having ≧4 fold lower HI titres with the mutant virus

Further analyses revealed that the 8 out of 30 sera (27%) from young children which failed to react with the R12 mutant (HI titres <10) had a GMT of 25 9 against the Tex variant (not presentet in Table 1) The corresponding figures for older children were 15/49 (31%) and 13 8 respectively Only 6 out of 125 sera (5%) from adultd did not give positive reactions with R12 having at the same time a GMT of 10 8 against Tex virus

It has been shown that young children respond to i initial infection or vaccination by producing predomin tely strain specific (SS) antibodies to the HA antigen influenza virus Primed adults however usually respon by making cross reactive (CR) antibodies being able t react with a range of viruses within the subtype as we as SS antibodies directed against the first encountere member of the subtype (9 10 *Oxford et al* i preparation) Furthermore the SS antibodies have higher capacity to neutralize virus (5 11) to inhibit vir haemagglutination (4) and to modify viral replicatio (5) suggesting that young children with SS antibod could be a candidate group for the selection of ne antigenic variants showing »antigenic drift« away fro the previous prevalent strain and causing influenz epidemics at frequent intervals The findings presente here are consistant with the previously reported distribu tion of SS and CR antibodies among various age groups since young children in contrast to adults reacted to lo HI titres with the mutant strain R12 having at the sam time relatively high HI titres against the latest epidem H3N2 variant It is likely that the SS antibodies ma provide the most successful immunological pressur necessary for the selective growth of mutant viruses W have in another study (3) demonstrated that S antibodies from hyperimmune goat serum were highl

e in selecting new antigenic variants both *in vitro* and *in vivo* (mice) This special role of SS es was also suggested by *Virelizier et al* (12) present report makes it likely that sera from children could provide at least theoretically the favourable growth conditions for new antigenic s Several passages in individuals each having a nt and restricted anti HA antibody repertoire may essary in order to select a variant sufficiently ically different from previous strains to become nologically relevant as has been suggested by *ll et al* (15) and having the potential of spreading groups other than the very young Our mutant assumed to have mutated at one antigenic locus pond ng to the antigenic site recognised by the clone 185/1 may not have such an epidemic al since adults had comparable levels of antibo both the wild Type A/Texas and the antigenic t

as surprising that our mutant having undergone passage with only one monoclonal antibody ation should react so poorly with children s sera y therefore be assumed that the antigenic site ised by monoclone 185/1 could be an important or the primary human immune response to A/ /1/77 virus Similar studies are now in progress other anti Texas HA monoclonal antibodies in to investigate whether changes at other antigenic an be detected by human sera

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upply of mouse monoclonal antibody 185/1 by Dr r G *Webster* St Jude Children s Hospital Memphis Tenn and helpful discussions with Dr *Geoffrey C Schild* National Institute for Biological Standards and Control London are gratefully acknowledged Excellent technical assistance was provided by *Paal H Berg*

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